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EXPERIMENTAL PATHOLOGY

FURTHER EXPERIMENTS WITH FIXATION AREAS, BEARING ON THE PATHOGENICITY OF *BACILLUS COLI* IN PERITONEAL INFECTIONS.

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*From the Hale Clinical Laboratory, London Hospital.**

Received for publication March 12th, 1924.

In a previous paper by this writer (1921) it was shown that organisms of the colon typhoid group will leave the blood-stream and infect areas of inert matter (sterile gum) introduced into the subcutaneous tissues, while certain other bacteria do not possess this power of migration. Similar experiments relating to coliform infections of the peritoneal sac are recorded in this paper, including some relating to adult rabbits that had been first inoculated with *B. coli.*

EXPERIMENTS WITH GUM.

Experiment 1.—Showing the degree of pathogenicity when the living organism is injected directly into the peritoneum.

2500 million living *B. coli* from a 24-hour culture were suspended in saline and injected into the peritoneum of each of two rabbits, neither showed any ill-effect. One was killed 24 hours later. Post mortem the peritoneum was a little injected, and contained a little free fluid in which were some exfoliated endothelial cells and a few leucocytes, with a small number of coliform bacilli, mostly very degenerate and staining poorly. Cultures grew a few colonies of *B. coli.* Cultures from the heart's blood remained sterile.

The experiment shows that the peritoneum rapidly deals with a considerable number of living *B. coli* by a bactericidal process, and that when so administered they have very little pathogenic effect. The living bacteria do not escape from the peritoneum into the blood-stream.

Experiment 2.—Pathogenicity from direct inoculation into the blood-stream.

A living culture of 2500 million *B. coli* was inoculated into the blood-stream of 2 full-grown rabbits. There were no obvious ill-effects. In both instances the bacteria were recovered from the blood in culture up to 72 hours after inoculation, but not later. A third animal, having received the same injection,

* Work carried out under a grant from the London Hospital Research Fund.

was killed 18 hours afterwards. No abnormality was noted in the peritoneal cavity, and a free swabbing of the peritoneal surfaces produced no growth in culture.

Experiment 3.—Effect of gum on the peritoneum.

Two rabbits were injected intraperitoneally with 5 c.c. of sterile 5% gum tragacanth. Neither showed any ill-effect. One was killed 24 hours later. Post-mortem the peritoneum was a little injected, and contained a little free gummy fluid in which a few leucocytes and endothelial cells were present. No organisms were seen and cultures were sterile.

Gum, then, leads to a little mild irritation of the peritoneum.

Experiment 4.—Inoculation of gum into the peritoneum and living bacteria into the blood-stream.

Two rabbits were inoculated with 5 c.c. of sterile gum into the peritoneum and 2500 million *B. coli* into the blood-stream. Neither showed any ill-effect. One was killed 18 hours after injection. Post-mortem the peritoneum was much as in Experiment 3, but though no bacilli were seen in films, a few colonies of *B. coli* grew in culture.

It appears from this experiment that the bacteria do penetrate from the blood-stream into the gum in the peritoneum, but apparently their numbers are not sufficient to set up an acute inflammation.

Experiment 5.—Direct inoculation of bacilli mixed with gum into the peritoneum.

2500 million living *B. coli* mixed into 5 c.c. of sterile gum tragacanth were injected into the peritoneum of each of two rabbits. One animal died 18 and the other 36 hours later with acute toxic symptoms. In the former the peritoneum was injected and showed some free, turbid, gummy fluid. Flms showed a few leucocytes and endothelial cells and enormous numbers of *B. coli*, which gave a massive growth on culture. *B. coli* also grew from the heart's blood. In the latter animal the peritoneum was somewhat injected, but in addition there was an enormous congestion of the large bowel and Fallopian tubes. This congestion did not affect the small bowel, although there was a little lymph adherent to this as to the large bowel. A massive growth of *B. coli* was grown from the peritoneal fluid, and also from the heart's blood.

In this experiment the bacteria have been protected from the destructive powers of the peritoneum and have multiplied and played an immensely pathogenic *rôle*. The bacilli have also escaped from the peritoneum into the blood-stream.

The localization of the congestion in the large bowel and organs of generation in spite of the generalized nature of the peritonitis suggests a central rather than a peripheral injury—possibly an injury to the centres controlling the vaso-motor nerves to these parts. Certain bacilli of the colityphoid group do, of course, localize in specific parts of the bowel, and this preliminary congestion might explain why they do so.

Experiment 6.—Intraperitoneal injection of dead bacilli with gum.

Two rabbits were injected intraperitoneally with 5000 million *B. coli* (killed by heating to 56° C. for 2 hours) mixed with 5 c.c. of sterile gum. Neither animal showed any ill-effect.

The experiment shows that the fatal effect following the injection of living organisms in gum is due either to their rapid multiplication, and the production, following lysis, of a very large amount of toxic substance, or else to the actual products of their life processes.

Experiment 7.—Showing that there is no great increase of pathogenicity after a single passage.

One c.c. of turbid peritoneal fluid from the first of the animals dying in Exp. 5 and containing abundant living *B. coli* was injected into the peritoneum of a fresh animal. No ill-effect was noted, and cultures from the blood-stream remained sterile.

EXPERIMENT WITH BILE.

In experiments on the pathogenicity for rabbits of *B. typhosus*, Besredka (1919) has been able to communicate the infection to rabbits by treating the intestinal canal with bile; in the same way he has also been able to achieve a local immunity of the intestinal mucosa against the infection. The fact that bile has a great effect in lowering surface tension makes it probable that it alters the cell membranes so as to render them permeable to the organisms. It is used with that intention in the following experiments.

As a preliminary rabbits were inoculated with 2 c.c. of ox bile intravenously and the same amount intraperitoneally. No ill-effect was noted. A further rabbit was given by the blood-stream 2 c.c. of bile mixed with 2500 million living *B. coli* in saline. This animal also sustained no harm.

These preliminary experiments show that the bile itself in the blood-stream or in the peritoneum is not harmful, and that its presence in the blood-stream along with living bacteria does not obviously add to their pathogenicity.

Experiment 8.—A repetition of Exp. 4 with the addition of bile.

A rabbit was injected intravenously with 2500 million living *B. coli* and 2 c.c. of bile, and at the same time 5 c.c. of sterile gum was injected into the peritoneal cavity. The animal died with acute toxic symptoms in 18 hours. The peritoneum contained a small excess of slightly sanious fluid showing numerous endothelial cells and polymorph leucocytes. Enormous numbers of living *B. coli* were present.

The experiment suggests that the passage of bacilli from the blood-stream into the fixation substance in the peritoneum has been greatly facilitated by the presence of the bile in the blood-stream.

In a complementary experiment the bile was put with the gum into the peritoneum and the living organisms into the blood-stream, and, as before, a fatal result rapidly followed.

EXPERIMENTS ON PREVIOUSLY IMMUNIZED ANIMALS.

The animals were treated with intravenous injections of living *B. coli*, first 2500 million, and a week later 5000 million. After the second injection living bacteria were still recovered from the blood-stream for 18 hours, but not subsequently, in all 3 cases. The destructive power in the blood-stream is evidently much greater in these inoculated animals than in the normal animal (*cf.* Exp. 2); on the other hand, no increased bactericidal effect of the blood-

serum of these inoculated animals over the normal animals was demonstrated in a series of experiments *in vitro*.

The agglutination titre of the inoculated animals for *B. coli* was upwards of 1/2560 (56° C. for 2 hours—macroscopic).

Experiment 9.—Repeating Exp. 5, but with larger doses.

Two of the immunized rabbits were inoculated into the peritoneum with 5000 million living *B. coli* in 7 c.c. of gum; neither showed any ill-effect. One was killed 24 hours later. Post mortem the peritoneum contained some turbid gummy fluid, with a few leucocytes. No organisms were seen and cultures were sterile.

Experiment 10.—Repeating Exp. 7 on 2 immunized animals.

7 c.c. of gum was injected into the peritoneum and 5000 million living *B. coli* plus 2 c.c. of bile into the blood-stream; no ill-effect followed, and in one animal which was killed 24 hours later the peritoneal fluid was sterile. The heart's blood also was sterile.

In Exps. 9 and 10 it is clear that the previous inoculations have protected the animals against what would otherwise have been a fatal infection.

DISCUSSION.

Among the functions of the gums in nature is that of affording a protective covering, especially for various types of seeds, spores, etc., and there can be little doubt that the mucus of the body with its somewhat similar physical characters functions in this way in the intestines, acting as a buffer between the bowel wall and the masses of contained bacteria. On this assumption gum was used in the experiments published previously by this writer (1921), where it was shown that bacteria embedded in gum and introduced into the tissues could preserve their vitality for several weeks, and this was true even of such fragile organisms as Shiga's dysentery bacillus (Benians, 1920). Later experiments, unpublished, have also shown that in animals immunized with *B. coli*, these organisms buried in gum in the tissues still remain viable just as they do in a non-immunized animal.

In the experiments described in this paper gum and bacteria are put into the peritoneum and here the mechanical conditions are quite different. The gum, instead of remaining in a mass as it does in the tissues, is diluted and spread out in a thin layer over the membrane, and leads to some irritation or mild inflammation, and while the serous membrane gains no protection, but rather a loss of vitality, the bacteria in the peritoneum of the non-immune animal are protected from peritoneal lysis and enabled to multiply. That the bacteria can derive protection from the gum, whilst the serous membrane cannot, argues an inherent property of the bacteria probably situated in the cell envelope, which is perhaps itself mucoid in nature. In the immunized animal it is doubtless this envelope which suffers a change, and renders the organism unable to resist the peritoneal lysis.

The penetration of organisms from the blood-stream into the peritoneum and their protection there by the gum may throw some light on the problems of blood-borne peritonitis, and of pneumococcal and streptococcal peritonitis, whether arising by blood infection or by direct invasion through the genital tract in females. One cannot argue direct from *B. coli* to the streptococcal

organisms, since these latter have a greater inherent pathogenicity for man, but it is highly improbable that a few organisms arriving in the blood-stream would gain access to the peritoneum without some preliminary irritation or injury of the membrane, or that they would survive peritoneal destruction without some protection in the peritoneum, so that a preliminary extravasation of mucus from some source would most easily explain a peritoneal infection arising apart from a perforation. Again, in the pneumococcal infections of the peritoneum which occur in young girls, McCartney and Fraser (1922) have traced the pneumococcus from the external genitals, and Bond (1913) has shown that there may be reflux currents of mucus in the female genital tract, so that in these young children the peritoneum might have no acquired immunity, and the pneumococcus might be protected by mucus, which conditions would supply all the requirements for an acute infection.

The action of bile in aiding the migration of bacteria is also of interest. The relation of jaundice to some severe septicæmias, especially fatal terminal infections, raises the question as to whether the jaundice is not the primary change, the circulation of the bile then setting free the bacteria into the blood-stream.

CONCLUSIONS.

1. The peritoneum of the normal rabbit has the power of rapidly killing large numbers of living *B. coli*, and no toxic effect follows. The destruction is effected apparently by a process of lysis. Phagocytosis is not observed to play an active part.

2. The protective mechanism is upset by the presence with the living (but not with dead) bacilli of gum tragacanth, which protects the bacteria, and they multiply and kill the animal in a few hours.

The toxic effect, if not too rapid, appears to be associated with a selective congestion of the large bowel and female organs of generation.

3. If gum is put into the peritoneum and *B. coli* into the blood-stream the bacilli will infect the peritoneum, although not in sufficient numbers to set up an acute disease.

4. In the immunized animal the peritoneum is quickly sterilized even if large numbers of living bacteria are present in gum. In such animals the bacteria survive a far shorter time in the blood-stream than in normal animals, but no increased bactericidal power of the serum was demonstrated *in vitro*.

5. The presence of bile in the blood-stream with living bacteria leads to a rapid invasion of gum in the peritoneum and a fatal infection in the non-immunized animal, but previously inoculated animals survive it easily.

6. In certain circumstances *B. coli* in the peritoneum may be immensely pathogenic.

7. The advantage of a preliminary immunization with *B. coli* as a protection against infection with this organism is demonstrated.

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ON THE GLYCOGENIC FUNCTION OF THE LIVER AND ITS ENDOCRINE CONTROL.

W. CRAMER.

From the Laboratories of the Imperial Cancer Research Fund, London.

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In the course of previous investigations (Cramer and Krause, 1913; Cramer and McCall, 1916, 1918) on the relation of the thyroid gland to carbohydrate metabolism, a number of apparently paradoxical phenomena appeared which were confirmed by Kuriyama (1917) and by Sanger (1921). It was pointed out at the time that these facts demanded a revision of our conception of carbohydrate metabolism. We emphasized in particular two points: firstly, that the glycogenic function of the liver is of greater importance than is generally supposed, and secondly, that the cells respond to an increased supply of carbohydrates by an increased metabolism. These views have been confirmed by further investigation on the relation of the activity of the thyroid and adrenal glands to metabolism. The recent work on insulin (for literature see MacLeod, 1924) has thrown light on a new aspect of carbohydrate metabolism, which presents striking similarities and equally striking dissimilarities when compared with the condition of carbohydrate metabolism in experimental hyperthyroidism.

This paper is an attempt to correlate the observations on the relation of different endocrine organs to carbohydrate metabolism, and to resolve the apparent contradictions which have arisen as a result of a mistaken conception of the glycogenic function of the liver.

Both the thyroid and the pancreatic hormones when administered to normal animals in sufficiently large doses induce a disappearance of glycogen from the liver. There is also an increased consumption of carbohydrates. But there the similarity ends, and the outstanding effect on carbohydrate metabolism just referred to is associated with entirely different effects on the other aspects of metabolism. Thus with a large dose of insulin the blood-sugar falls, but no such effect is produced by thyroxin or by prolonged thyroid feeding. Thyroid feeding produces, as McCall and I showed, an increased transformation of protein and possibly fat into carbohydrates and increased nitrogen excretion, and an increased metabolism generally as indicated by an increased oxygen consumption. There is also a rise in the body-temperature, as the accompanying chart shows (Chart 1). The effect of insulin is in the opposite direction: the nitrogen excretion falls, the temperature is subnormal, the oxygen consumption either does not change or declines, so that the general effect is to reduce metabolism (Macleod, 1924). While, therefore, the thyroid and pancreatic hormones both increase the consumption of carbohydrates by the cells, their effect on protein metabolism is exactly the opposite; with the

pancreatic hormone the protein-sparing action of carbohydrates comes into evidence, with the thyroid hormone increased protein destruction. In fact, so far as protein metabolism is concerned we find that the absence of insulin in pancreas diabetes and the resultant impaired oxidation of carbohydrates is associated with the same increased protein destruction and nitrogen excretion, which sets in also as the result of increased activity of the thyroid hormone, either when produced by feeding with thyroid or in Graves's disease. Even the nitrogen distribution in the urine is similar in hyperthyroidism and in pancreas diabetes (Cramer and Krause, 1913). The most paradoxical feature is to be found in the liver glycogen, which is diminished both when the pancreatic hormone is deficient and when it is acting in excess, and also when the thyroid hormone is acting in excess.

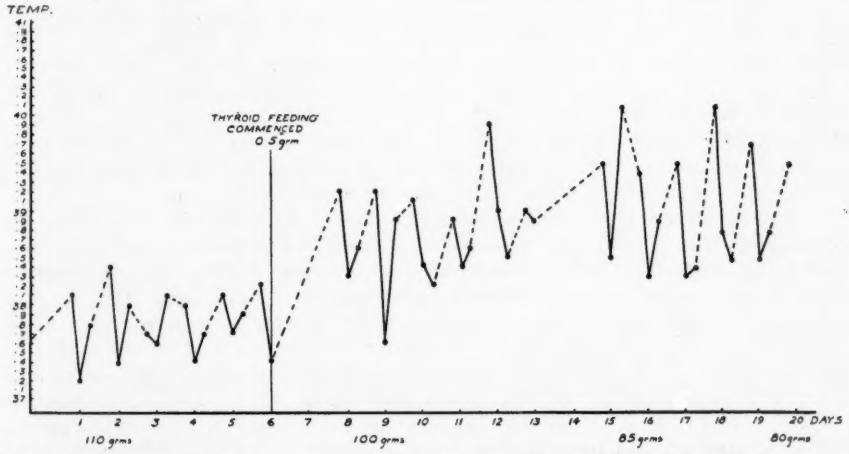

CHART 1.—Temperature curve of rat before and after thyroid feeding. The temperatures were taken three times a day—at 10 a.m., 1 p.m., and 5 p.m. Note that thyroid feeding produces not only a rise in temperature but also a "hectic" type of fever. Two other rats gave similar results.

The effect of an injection of adrenalin is too transient to produce marked effects on metabolism, especially if the dose is kept within physiological limits. But if by stimulation of the adrenal medulla a prolonged adrenalinæmia is induced—as can be done by the injection of β-tetrahydronaphthylamin—the metabolism is profoundly affected. The metabolic changes are in the same direction as those observed in hyperthyroidism but they are more acute: there is disappearance of glycogen from the liver, hyperglycæmia, increased oxidation of carbohydrates, increased nitrogen excretion and increased heat production (for literature, see Cramer, 1920). The condition differs from hyperthyroidism in the active constriction of the peripheral blood-vessels, which leads to diminished heat loss. As a result there is a rapid rise in temperature and the condition of "sympathetic fever" is established.

For convenient reference the metabolic changes in these various conditions

are summarized in the following table, in which + stands for an increase above the normal and − for a diminution.

	Liver glycogen.	Blood-sugar.	Consumption of carbohydrates.	Glycosuria.	N. excretion.	Temperature.
Hyperthyroidism	−−	+	+	−	++	+
Hyperadrenalinism (*sympathetic fever*)	−−	+	+	−	++	+++
Hyper-insulinism	−	−−	+	−	−	−−
Hypo-insulinism (*diabetes mellitus*)	−−	+++	−−−	+++	++	Normal

The disappearance of glycogen from the liver would appear, therefore, to have a different significance in these different conditions. This conclusion is confirmed when one compares the effect of insulin on a fasting animal with that on an animal in a condition of hyperthyroidism. In both these conditions the liver is free or almost free from glycogen. But while the fasting animal is much more sensitive to insulin—as indicated by the hypoglycæmic collapse—than a fed animal, experiments on rats have shown that hypoglycæmic collapse cannot be induced more readily in a hyperthyroid rat after a meal with the liver free from glycogen than in a normal fed rat.

The explanation usually given for the greater sensitiveness to insulin of a fasting animal is that under the effect of insulin the cells absorb more sugar from the blood, that the blood-sugar is replenished by the liver glycogen so long as this store of carbohydrates is available and falls when this store is exhausted, until when a certain low level of blood-sugar is reached hypoglycæmic collapse ensues. This explanation is, however, not an adequate one. It fails to take into account the existence of other conditions, where there is an increased oxidation of carbohydrates associated with the disappearance of glycogen from the liver without hypoglycæmia occurring. In experimental hyperthyroidism the liver is free from glycogen and the oxidation of carbohydrates is increased. But in that condition not only is there no hypoglycæmia, but a rise of blood-sugar above the minimal level, and instead of a hypoglycæmic collapse with lowered body-temperature we have an increased metabolism with a higher body-temperature. Similarly the drug tetrahydronaphthylamin produces, as has been stated, a prolonged adrenalinæmia and general sympathetic stimulation with such active mobilization of glycogen that the liver becomes glycogen-free. There is also in this condition an increased oxidation of carbohydrates as indicated by a rise in the respiratory quotient. The result is, however, not hypoglycæmic collapse with a subnormal temperature and lowered metabolism, but hyperglycæmia and increased metabolism with hyperpyrexia—a condition which we have called " sympathetic fever " (Cramer, 1920). And if, in order to accentuate the association of increased oxidation of carbohydrates with a glycogen-free liver, we super-impose the effect of tetrahydronaphthylamin upon an animal in a condition of experimental hyperthyroidism, we find again that hypoglycæmic collapse does not set in. On the contrary, since thyroid feeding and tetrahydronaphthylamin have a synergetic effect, a thyroid-fed animal is rendered more sensitive to the action

of tetrahydronaphthylamin, so that a dose of this substance so small as to be ineffective in a normal rat will kill the same animal in hyperpyrexia, if it has been subjected previously to thyroid feeding for a few days. We have here, therefore, a summation of stimuli. A previous injection of insulin has the reverse effect. A dose of tetrahydronaphthylamin which will kill a normal rat in hyperpyrexia fails to elicit sympathetic fever when injected one hour after the injection of a dose of insulin, even when the dose of insulin is insufficient in itself to produce the hypoglycæmic collapse. This was observed in eight out of ten rats. In eight of these the temperature fell, and only two developed hyperpyrexia. All the rats died with the lesions which are found after lethal doses both of tetrahydronaphthylamin and of adrenalin—hæmorrhages into the lungs or brain. The stimulation of the adrenals by tetrahydronaphthylamin to secrete adrenalin is therefore not inhibited by the preliminary injection of

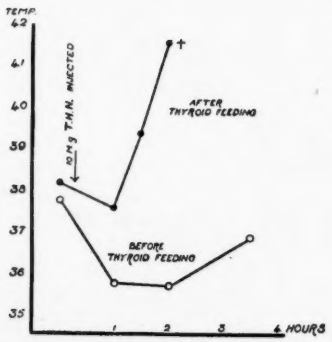

CHART 2.

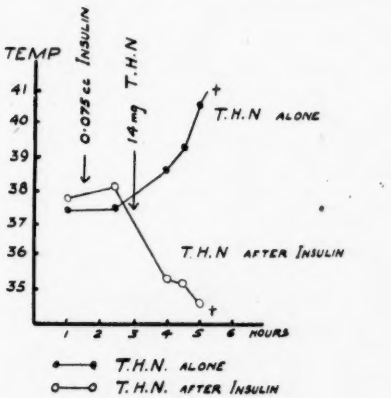

CHART 3.

CHART 2.—Effect of thyroid feeding on response to tetrahydronaphthylamin ("T.H.N.") A dose of T.H.N. which in a normal rat was insufficient to produce a fever and gave only a fall in temperature, killed the same animal in hyperpyrexia after it had been fed on thyroid gland for five days.

CHART 3.—Effect of insulin on response to T.H.N. A dose of T.H.N. which killed a normal rat in hyperpyrexia, produced in another rat which had received a dose of insulin insufficient in itself to elicit hypoglycæmic collapse a rapid fall of temperature below 35° with collapse in which the animal died.

insulin. The antagonism between insulin and tetrahydronaphthylamin is in fact only a manifestation of the well-known antagonism between insulin and adrenalin. Charts 2 and 3 represent typical experiments.

All these observations go to show that the absence of glycogen after thyroid feeding on the one hand and after insulin on the other represents two entirely different functional states of the liver. This conclusion is confirmed by the histological appearance of the liver-cells in these two conditions, as the three accompanying drawings show.

These represent the livers of mice fixed in Schridde's fixative (formol-Müller followed by osmic acid) for the demonstration of mitochondria and

stained with Heidenhain's hæmatoxylin. A normal liver-cell (Fig. 1) when prepared in this way shows its cytoplasm filled with densely staining granules—the mitochondria. After thyroid feeding (Fig. 2) the granular nature of the liver cytoplasm is maintained, and the most obvious deviation from the normal is the appearance of fine globules of fat. These globules are larger than the mitochondria and are blackened by the osmic acid of the fixative. After insulin (Fig. 3) the cytoplasm is profoundly altered, and shows regressive changes: the mitochondria have disappeared, and the cytoplasm, instead of having a granular appearance, has a colloid appearance as if the cytoplasm had become confluent.

GENERAL DISCUSSION.

What, then, is the significance of these two different states? The key to the answer is to be found in the behaviour of the blood-sugar in the different conditions when the glycogen disappears from the liver.

The blood-sugar percentage of the normal animal is variable and varies with every intake of carbohydrate. But it never falls below a certain minimal level, even in prolonged fasting, although the glycogen may be absent from the liver. Only when the liver is removed or severely damaged does the blood-sugar fall below that minimal level, and then the animal enters into a state of collapse, which can be relieved at once, though only temporarily, by the injection of glucose (Mann and Magath, 1923). One function of the liver is therefore to form carbohydrate autonomously and from non-carbohydrate material, when no carbohydrate is supplied in the food, and to pass it into the blood in the form of glucose automatically whenever the level of the blood-sugar reaches a certain minimal level, so as to prevent the blood-sugar from falling below that level. As we shall see presently, this autonomous formation of carbohydrate—and not the mere deposition of glycogen when an excess of carbohydrate is supplied in the food—should be recognized as constituting the glycogenic function of the liver. When insulin is injected the blood-sugar falls below the minimal level: the effect is the same as if the liver had been temporarily removed or damaged. This suggests that insulin, in addition to inducing an increased consumption of glucose by the tissues, also inhibits the autonomous formation of carbohydrate by the liver. In hyperthyroidism the blood-sugar fluctuates above the minimal level, although the liver remains free from glycogen. This suggests that in hyperthyroidism the glycogenic function of the liver is stimulated. In conjunction with McCall, I have adduced evidence that in experimental hyperthyroidism the rate of formation of carbohydrate and of its secretion is increased. We pointed out there that the amount of glycogen present in the liver represents merely the balance between the rate at which carbohydrate is formed and that at which it is passed into the blood, and is not a measure of the glycogenic function of the liver—in other words, one should look upon the glycogen of the liver dynamically, and not, as has been done, statically as a mere store of excess of carbohydrate.

This dynamic conception is not new. It was, as we shall see presently, the conception of Claude Bernard. It was replaced by the static conception of the liver glycogen as the result of a mistaken conception of carbohydrate metabolism generally. The conception of carbohydrates to which we refer

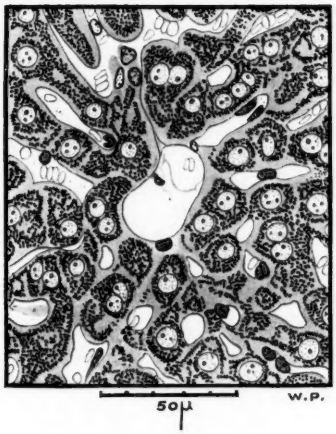

FIG. 1.

Liver of normal mouse, fasting for two days, fixed in Schridde's fixative and stained with Heidenhain's hæmatoxylin for mitochondria. × 420. The cells, which contain little glycogen, show a cytoplasm packed with densely staining mitochondria.

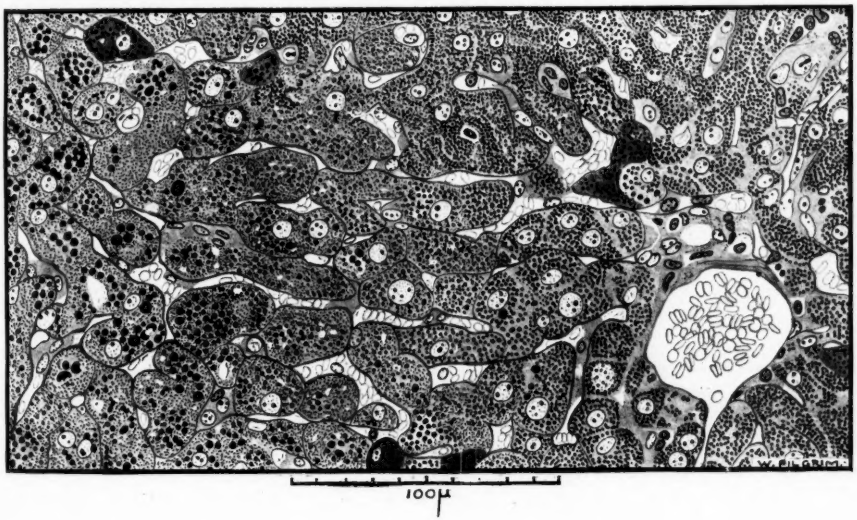

FIG. 2.

Liver of mouse fed with dried thyroid gland for four days. Fixed and stained in the same way. × 420. The cells contain little glycogen; their cytoplasm, like that of the normal mouse, is packed with mitochondria. The left side of the figure shows cells containing globules of osmic stained fat. These are larger than the mitochondria, and are, of course, visible in the unstained preparation as black globules.

Cramer.

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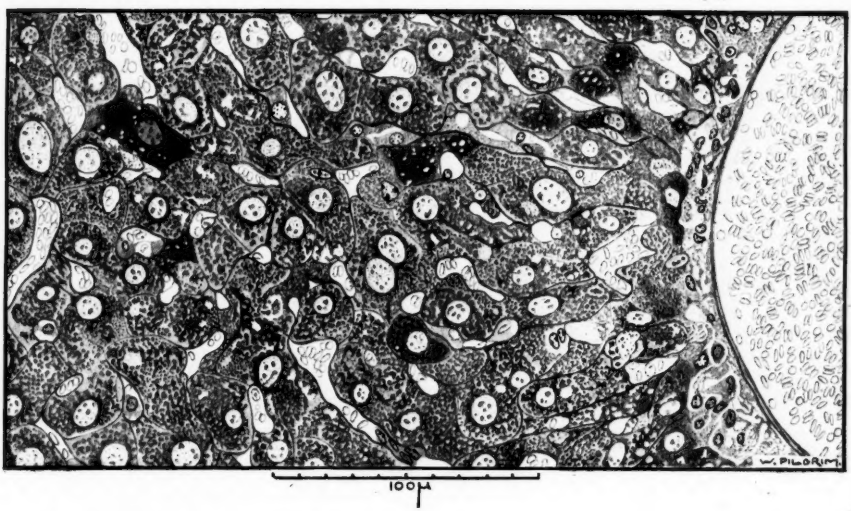

FIG. 3.

Liver of mouse two hours after a dose of insulin which had produced hypoglycæmic collapse. Stained and fixed in the same way. × 420. The cells still contain some glycogen, indicated by the vacuoles. The mitochondria have disappeared and the cytoplasm has become confluent.

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was formulated when it was believed that the blood-sugar had a constant level. Its three cardinal tenets were :

1. That the blood-sugar percentage is constant.

2. That the function of the liver is to keep it constant by storing an excess of carbohydrate as glycogen and thus preventing an excess from entering into the systemic circulation.

3. That the oxidation of carbohydrates is conditioned entirely by the needs of the cells.

This conception is tainted with teleology. Everything is explained as a means to an end, namely, to maintain the level of the blood-sugar constant. When the refinements of micro-chemical technique showed that this end was not attained, since the blood-sugar percentage may double after a meal rich in carbohydrates, the whole conception was deprived of its basis and ought to have been abandoned. But it still dominates the current view of carbohydrate metabolism. Many writers still speak of the blood-sugar as being " practically " constant, and are compelled to concede that the liver does not perform its function very efficiently. And the important significance of the blood-sugar variations is not yet generally appreciated.

To take the last point first. Pflüger, in his book on 'Glycogen' (p. 449), lays down as a fundamental principle of metabolism, that only the work of the organs determines the metabolism and not the amount of food offered to the organism, and he says further that this applies particularly to the metabolism of fats and carbohydrates. The conceptions of metabolism developed by Rubner and by Zuntz are based on the same principle. It is difficult to ascertain on what experimental evidence this dogmatic statement is based. But it was and—in spite of recent work to the contrary, especially the work of Lusk—still is generally held. Lusk (1912) showed conclusively that the inflow of sugar into the blood which occurs when glucose is given stimulates the cells to an increased oxidation of carbohydrates, which does not protect corresponding amounts of fat or protein from oxidation as was believed, but partly adds itself to them, so that the heat production is increased. In this respect, therefore, the supply of food material determines the metabolism of the cell. Lusk called this condition " the metabolism of plethora." Evans (1914), in showing that the oxygen consumption of the blood-perfused heart increased when glucose was added to the perfusion fluid, confirmed this conception for an isolated organ. My observations with McCall on the gaseous metabolism in experimental hyperthyroidism showed that in this condition the primary effect of the thyroid hormone is a mobilization of glycogen from the liver, presumably as the result of stimulation of the sympathetic, and that the resultant influx of carbohydrates into the blood leads at once to an increased oxidation of carbohydrates. If thyroid feeding was prolonged sufficiently there was also increased metabolism, increased protein destruction and increased heat production. The same stimulation of metabolism was found by me later to hold for other conditions of sympathetic stimulation, as, for instance, when a prolonged and massive secretion of adrenalin from the adrenal medulla is induced either by exposure of the animal to cold or by the injection of tetrahydronaphthylamin. All these conditions studied by me differ from the " metabolism of plethora " of Lusk in

having added to the factor of carbohydrate plethora the factor of sympathetic stimulation with the resultant increase in heart-beat and respiration and other changes. The increased heat-production in these conditions manifests itself by a gradual rise in the body-temperature in hyperthyroidism, by a hyperpyrexia after the injection of tetrahydronaphthylamin, while it enables the animal to maintain its normal temperature if exposed to cold. These observations, together with a number of other facts, formed the basis on which we formulated the view that the thyroid and adrenal glands, having a synergetic action on metabolism, form a humoral mechanism concerned in the heat regulation of the body (Cramer, 1916, 1919). These views have received ample confirmation by more recent work. Thus Hartman and his collaborators (1923) confirmed by means of the denervated pupil reflex that cold is a very effective stimulus for the excretion of adrenalin from the medulla. Aub and his colloborators (1922) emphasized that the action of adrenalin on metabolism is qualitatively the same as that of thyroxin. Boothby and Sandiford (1923) confirmed the fact that adrenalin increases the heat-production. On finding that the increase in heat-production is much greater after the injection of adrenalin than after the ingestion of glucose while the increase in blood-sugar is much greater in the latter condition, they postulated that adrenalin increases directly the oxidative power of the cells, and attributed this as a specific property of adrenalin, which they now call the " calorigenic action " of adrenalin.

It is clear from what has been said that the increase in heat-production after adrenalin had been demonstrated before. What is new in Boothby and Sandiford's work is their idea that this is due to a direct stimulation of the cells by adrenalin. This possibility had been tested before repeatedly by a number of observers, especially by Evans and Ogawa (1914), in observations on the heart-lung preparation. All are agreed that the increase in metabolism which ensues after adrenalin is due to the sympathetic stimulation increasing the activity of the cells (accelerated heart-beat, rapid respiration, contracted musculature of the blood-vessels), and that no direct action of adrenalin could be demonstrated. Nor has the more recent work of Boothby and Sandiford adduced any evidence which would substantiate their contention. The increased heat-production after sympathetic stimulation can be adequately explained by the two known factors—the increased muscular work and cellular activities resulting from sympathetic stimulation, and secondly, the influx of carbohydrates from the liver, which at once provides suitable fuel material to maintain these increased activities.

We may now turn to the conception that the liver glycogen is a store of the excess of carbohydrates. As we have seen, this view formed part and parcel of the conception of the constancy of the blood-sugar level as an essential and important feature of carbohydrate metabolism. It ought logically to have been abandoned when it was found that the liver failed to keep the level of the blood-sugar constant. The difficulty was got over by conceding with some indulgence that the liver did not perform its function very efficiently. Having perceived its failure the liver is supposed to rouse itself somewhat belatedly, and by withdrawing the " excess " of sugar from the blood to "regain control." This conception illustrates the firm hold, amount-

ing almost to a dogma, that the idea of the constancy of the blood-sugar had obtained.

The complete failure of this conception of the liver glycogen was demonstrated when it was found that as the result of thyroid feeding the liver ceased to store glycogen. If the accepted view of carbohydrate metabolism was correct an intense alimentary glycosuria should have occurred on a diet rich in carbohydrates. But this did not happen. The urine did not even reduce Fehling's solution. Instead there was an increased oxidation of carbohydrates. But this again did not protect, as one would have expected, protein from oxidation; on the contrary it led to an increased oxidation of protein. The nitrogen excretion in the urine rose, and its distribution gave a picture identical with that obtained in diabetes mellitus or when carbohydrates are completely withdrawn from the diet, although the animal was living on a diet rich in carbohydrates. The respiratory quotient showed the same paradoxical behaviour; even immediately after a meal rich in carbohydrates it gave low values, which in a normal animal would be interpreted as an inability to oxidize carbohydrates. The facts remain incomprehensible and paradoxical only so long as we adhere to the current view of carbohydrate metabolism. They fall into an ordered and easily comprehended sequence as soon as we free ourselves from preconceived conceptions and allow ourselves to be guided by facts. I shall refer first to facts which have long been known.

Glycogen does not permanently disappear from the liver as the result of withholding carbohydrates, nor does it disappear permanently even in the course of prolonged fasting. On the contrary under such conditions the liver actively forms glycogen. The liver may be made almost free from glycogen by inducing by means of strychnine violent muscular contractions in a fasting animal. If the effect of strychnine is allowed to pass off and the fasting is continued, glycogen will again make its appearance in the liver. Another striking example is reported by Pflüger, who found 24·3 gm. glycogen (=4·8 per cent.) in the liver of a dog which had been kept without food for 28 days. The glycogen content of the liver of a number of normal well-fed dogs is given by Pflüger as varying between 4·3 and 18·7 per cent. The effect of starvation was much more pronounced in the glycogen content of the muscular tissue, which amounted only to 0·16 per cent. in starved animals as against 0·7 to 3·7 per cent. of glycogen found in the muscles of well-fed dogs. Claude Bernard showed that the liver-cells have the power to form carbohydrate material from protein, and do so when no supply of excessive carbohydrate in the food is available. A consideration of these facts leads to the conclusion that glycogen represents the specific product of the autonomous secretory activity of the liver-cell, in the same way as trypsinogen, for instance, is the product of the pancreatic cells and adrenalin that of the medullary cells of the adrenal.

The nature of glycogen as a specific secretory product of the hepatic cells which is being secreted into the blood-stream automatically in small amounts when the blood-sugar reaches a certain minimal level, and more massively as the result of stimulation of the gland, cannot be described more succinctly than by calling glycogen "the internal secretion of the liver." And there is a very good precedent for doing so. For it was Claude Bernard, the discoverer of

glycogen, who coined the words "internal secretion" in order to describe the functions of glycogen (see Appendix). This term "internal secretion" was not a happy and accidental inspiration on his part. To Claude Bernard the glycogen of the liver was essentially a product of secretion, an autonomous function of the liver, and not merely a store of excessive carbohydrate from the food. For he showed that glycogen could be formed from protein entirely even in the absence of carbohydrates, though, contrary to statements attributed to him erroneously in text-books, he did not deny that it could also be formed from carbohydrates, if these were supplied in abundance.

One cannot but admire the intuition with which Claude Bernard recognized the fundamental character of the glycogenic function, and one cannot but regret that when his views were generally accepted, as the text-books say they were, the most important part of them was allowed to be forgotten.

The liver glycogen conforms even to the recent definition of an internal secretion given by Swale Vincent (1922). He defines the process of internal secretion as "the preparation and setting free of certain substances of physiological utility (the raw materials for which are supplied by the circulating blood) by certain cells of a glandular type; the substances set free are not passed out on to a free surface but into the blood-stream." There are obvious differences between such a substance as glycogen and such substances as adrenalin, thyroxin, or other as yet chemically unknown internal secretions. The differences are so obvious that the point need not be laboured. They are not likely to be overlooked, or forgotten. What is less obvious, and what has been overlooked since Claude Bernard pointed it out, is the endocrine aspect of the glycogenic function of the liver, which is the key to an understanding of carbohydrate metabolism in its relation to the metabolism of proteins and to various endocrine organs.

In the light of this conception the glycogenic function of the liver appears as an autonomous secretory function, which is continuously active, proceeds independently of a supply of carbohydrates in the food, and if their supply is not adequate, draws upon proteins and possibly also fats for the formation of carbohydrate by the liver-cells. The presence of abundant carbohydrates in the food protects, therefore, protein from being used by the liver-cells, so that the nitrogen excretion in the urine is diminished, while conversely in the absence of carbohydrates the nitrogen excretion rises. This phenomenon, which is generally known as the "specific protein-sparing effect of carbohydrates," is not in evidence when we consider the metabolism of isolated organs, but is the result of the glycogenic function of the liver, which integrates the carbohydrate metabolism of the individual organs and tissues and brings it into relation with protein metabolism.

The study of the adrenal medulla has shown that stimulation induces not only a secretion of adrenalin but also an increased formation of adrenalin, so that the load of adrenalin present in the medulla is not a measure of the functional state of the adrenal medulla, but represents the balance between the rate at which adrenalin is formed and that at which it is secreted. In the same way stimulation of the glycogenic function by thyroid feeding, by adrenalin or by splanchnic stimulation involves not merely a secretion of carbohydrate—or, as it is usually called, a "mobilization" of glycogen—but

also an increased formation of carbohydrates; and the "load" of glycogen represents the balance between the rate of formation and of secretion of carbohydrate. If the stimulation is sufficiently prolonged, as in hyperthyroidism, the amount of carbohydrate formed by the liver-cells may be so great that the amount of pre-formed carbohydrate in the food is insufficient to cover the demands of the liver-cells, so that protein is being drawn upon by them for the formation of carbohydrate, which is secreted into the blood-stream as rapidly as it is formed. As McCall and I pointed out, all the metabolic features of hyperthyroidism—the increased basal metabolism with an increased nitrogen excretion, a glycogen-free liver and a low respiratory quotient with increased oxidation of carbohydrates—can thus be explained as the result of a hyperactive glycogenic function of the liver. If, conversely, we inhibit the formation of carbohydrate by the liver and at the same time cause the blood-sugar to fall by an increased consumption of carbohydrates by the tissues, the glycogen which disappears in an attempt to maintain the level of the blood-sugar is not replaced. The liver loses its glycogen, but its functional state is exactly the opposite to that obtained in hyperthyroidism. This is the condition produced by insulin. Since it is important to understand why we attribute to insulin an inhibiting effect on the glycogenic function—in particular on that aspect of this function which is concerned with the formation of carbohydrate — we may briefly recapitulate our reasons:

1. The inability of the liver to maintain the minimal level of the blood-sugar—a condition which occurs only after removal or severe damage of the liver.

2. The histological appearance of the liver-cells after insulin, which indicates a regressive change in their cytoplasm (disappearance of mitochondria).

3. The antagonism between insulin and agents known to produce a stimulation of the glycogenic function (adrenalin, tetrahydronaphthylamin), as compared with the combination of two stimulating agents producing a summation of stimuli (tetrahydronaphthylamin and hyperthyroidism).

On somewhat different grounds Lesser (1923) has recently arrived at the same conclusion, that insulin inhibits the formation of carbohydrate in the liver.

We see, then, that the glycogenic function of the liver is under the antagonistic influence of the pancreatic hormone, which inhibits it, and of the sympathetic system, which stimulates it, and which is itself subject to stimulation by the hormones of the thyroid gland and of the adrenal medulla.

But this antagonism does not extend to other aspects of carbohydrate metabolism, since all three hormones increase the consumption of carbohydrates by the cells, although not in the same manner. This conception is therefore quite different from that of the Viennese school. We can now also understand why the glycogen disappears from the liver, both when the pancreatic hormone is absent, as in diabetes mellitus, and when it is present in excess. While in the latter case it is due, as we have seen, to inhibition of the glycogenic function, in pancreas diabetes there is over-stimulation of this function. For when, after excision of the pancreas, the inhibiting influence of the pancreatic hormone is removed, the stimulating effect of the sympathetic

and of the sympathomimetic thyroid and adrenal hormones preponderates. The condition may be likened to the acceleration of the heart when the inhibiting influence of the vagus is removed. The glycogen-free liver of the diabetic organism corresponds therefore to the glycogen-free liver of the organism in hyperthyroidism. The increased nitrogen excretion in diabetes mellitus corresponds to the increased nitrogen excretion in hyperthyroidism with its similar nitrogen distribution in the urine. In both conditions it is a manifestation of the hyper-activity of the glycogenic function. The hyperactivity of the glycogenic function of the liver in diabetes mellitus was quite clearly recognized by Claude Bernard. In fact, he explains diabetes mellitus as the result of an increased sugar production by the liver. He writes ('Leçons sur le Diabète,' p. 437)—"le sucre est versé dans le sang en quantité anormale, d'ou hyperglycemie et glycosurie, mais la source hépatique n'est pas epuisée pour cela, elle continue à assimiler les matériaux propres à former le glycogène et par suite le sucre; elle redouble pour ainsi dire, d'activité pour remplacer le sucre éliminé, elle epuise l'organisme pour suffire à cette production, à cette dépense désordonnée en matieres sucrés." We know now that the hyperactivity of the glycogenic function and the resultant hyperglycæmia is not sufficient to produce glycosuria, unless there is also an impaired oxidation of glucose by the cells. But this latter factor has been considered too exclusively in recent work on diabetes mellitus and the hepatic factor has been unduly neglected. Insulin corrects both factors; it inhibits the hyperactivity of the glycogenic function and it facilitates the oxidation of carbohydrates by the cells. Experimental evidence is available which demonstrates the importance of the hepatic factor in diabetes mellitus. Recently Hendrix and Sweet (1923) have shown that the sugar excretion of a depancreatized dog is diminished when the liver is excluded from the circulation by an Eck's fistula. Similarly when by thyroidectomy in a depancreatized dog the thyroid hormone is prevented from stimulating the glycogenic function, the sugar excretion falls (Lorand, 1904; McCallum, 1909; Rohdenburg, 1920; Friedman and Gottesman, 1921, 1922). Conversely any condition which increases the functional activity of thyroid or adrenal should on these grounds aggravate the disease. We have shown in previous papers that cold is a powerful stimulant to these two glands, and it is a well-known clinical fact that exposure of a diabetic patient to cold increases the excretion of sugar and should be avoided. Administration of thyroid gland has also been found to aggravate the condition in diabetes mellitus. Lastly, the relation of insulin to the functional activity of the liver-cells affords an explanation of the pathological changes occurring in the liver as the result of the prolonged use of insulin in depancreatized dogs, described in the preceding number of this Journal by Macleod and his collaborators (Allan, Bowie, Macleod and Robinson, 1924).

SUMMARY.

The glycogen of the liver is not a reserve store of excess carbohydrate, but a product of secretion of the liver-cells—an "internal secretion" of the liver as defined by Claude Bernard. The glycogenic function of the liver is an autonomous secretory function, by which the liver forms carbohydrate in the

form of glycogen independently of any supply of pre-formed carbohydrate in the food and secretes it into the blood in the form of glucose. By means of this function the level of the blood-sugar is prevented from falling below a fixed minimal level, because the liver automatically secretes sugar when this level is reached. Another aspect of this glycogenic function is that by its stimulation more carbohydrate is formed and more glucose is secreted into the blood.

The specific protein-sparing action of carbohydrates in the general metabolism is due to carbohydrate protecting protein from being used by the liver for the formation of glycogen.

The glycogenic function is inhibited by the pancreatic hormone. It is stimulated by the sympathetic system and by the thyroid and adrenal hormones. This stimulation and inhibition affects both the formation of carbohydrate by the liver and the secretion of glucose into the blood. The antagonism between these two groups of hormones is restricted to the glycogenic function of the liver and not to the carbohydrate metabolism of individual cells or organs; in fact both groups of hormones increase the consumption of carbohydrates by individual organs although in different ways.

The inhibition of the glycogenic function of the liver is an essential factor in bringing about the hypoglycæmia after insulin. When the inhibiting influence of the pancreatic hormone is absent, as in pancreas diabetes, the glycogenic function of the liver becomes hyperactive. The hyperglycæmia of diabetes mellitus is the result not only of an impaired oxidation of glucose by the cells, but also of an increased formation of carbohydrate by the liver, and an increased secretion of glucose into the blood. The therapeutic effect of insulin in diabetes mellitus is directed not only towards improving the oxidation of carbohydrates by the cells, but also towards inhibiting the hyperactivity of the glycogenic function of the liver.

Stimulation of the glycogenic function by the thyroid and adrenal hormones leads in a normal organism to increased oxidation of carbohydrates, increased formation of carbohydrates from proteins, increased heat production and increased nitrogen excretion. It occurs in such conditions as experimental hyperthyroidism, Graves's disease, exposure to cold and sympathetic fever.

APPENDIX.

In view of its historical interest the following quotation from Claude Bernard's paper, in which the term "internal secretion" occurs for the first time, is given here ('Leçons de Physiologie experimentale,' Paris, 1855, p. 89):

"On s'est fait pendant longtemps une très fausse idée de ce qu'est un organe secréteur. On pensait que toute secrétion devait être versée sur une surface interne ou externe, et que tout organe secrétoire devait necessairement être pourvu d'un conduit excréteur destiné à porter au dehors les produits de la secrétion. L'histoire du foie établit maintenant d'une maniére très nette qu'il y a des *secrétions internes*, c'est à dire des secrétions dont le produit, au lieu d'être deversé à l'extérieur, est transmis directement dans le sang."

And twenty-two years later, in the 'Leçons sur le Diabète' (Paris, 1887), he

again applies to glycogen the term "secrétion." On p. 308 he says: "Ainsi la formation du sucre a pour condition préalable la production, la secrétion, du glycogène."

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ON THE BACTERIAL FACTOR IN THE ÆTIOLOGY OF DENTAL CARIES.

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ALTHOUGH many factors may enter into the ætiology of dental caries, there is little doubt that the lesions are the result of the action of bacteria. The initial process is one of decalcification, and Miller's theory—that this decalcification is produced by acid elaborated by the bacterial fermentation of carbohydrates in the mouth—is now almost universally accepted.

The object of the present investigation, which was undertaken at the instance of the Dental Diseases Committee of the Medical Research Council, was to ascertain if there was regularly present in early carious lesions a specific organism or group of organisms which could elaborate acid in sufficient quantity for the initiation of the process under conditions not very different from those obtaining in the mouth.

Goadby (1903) came to the conclusion that "dental caries is not a specific disease due to a specific micro-organism," but in more recent times Howe and Hatch (1917), and also McIntosh, James and Lazarus-Barlow (1922), have drawn attention to the fact that *Bacillus acidophilus* is constantly present in the carious lesions. McIntosh, James and Lazarus-Barlow produced lesions resembling those of caries in healthy teeth kept in glucose broth cultures of this bacillus, and this is the only direct evidence that has been produced up to the present to establish an ætiological relationship between a specific group of organisms and dental caries.

PRELIMINARY INVESTIGATIONS.

An attempt to determine the part played by *B. acidophilus*† in the ætiology of dental caries was first made. The teeth examined by Howe and Hatch, and McIntosh, James and Lazarus-Barlow were in a comparatively advanced stage of caries, with cavity formation, and the conditions in such cases are very different from those which obtain at the initiation of the disease.

* Working with a whole-time grant from the Medical Research Council.

† There is, apparently, a group of acidophile bacilli, the members of which, while differing from one another in minor points of morphology, in fermentation reactions and serologically, all have the general characteristics of the *B. acidophilus* of Moro. No attempt was made to analyse the group, and the name "*Bacillus acidophilus*" is used to designate any non-sporing, Gram-positive bacillus which produces acid in glucose broth and will grow on glucose agar with a reaction of pH 4.

The cavities, open to the mouth, retain considerable quantities of food material which rapidly ferments and becomes acid, and the resulting acidity encourages the growth of *B. acidophilus* while inhibiting that of other bacteria. The question to be decided was, therefore, whether *B. acidophilus*, which is a constant inhabitant of the mouth, is the cause of the carious lesions or merely a secondary invader of an already established process.

Technique of isolation.—Four teeth were in an advanced stage of caries, and in their case the technique of McIntosh, James and Lazarus-Barlow was adopted. The others were dipped momentarily into boiling water, in order to sterilize the surface as far as possible, wrapped in a piece of sterile sheet lead, placed in a vice and sawn with a sterile hack-saw, in most cases through a healthy portion, until it was thought that the base of the carious area had been reached, when they were split by inserting a sterile chisel into the saw-cut. The deepest part of the carious material was then scraped out with a sterile excavator and inoculated into glucose broths at pH 4 and pH 5·6.

Results.—Twenty-four teeth were examined, the majority being in an early stage of decay. *B. acidophilus* was isolated from 8, 4 of which were cases of advanced caries, while the other 4 had very shallow foci in the dentine with considerable erosion of the enamel, and it was impossible to obtain the material for culture without encroaching on the surface.

The above results indicated that *B. acidophilus* was not regularly present in the earlier lesions of caries. A second series of 24 teeth was, therefore, examined in order to confirm this, and at the same time to ascertain what organisms other than *B. acidophilus* could be isolated from such lesions.

The technique adopted was similar to that used in the previous experiments, but the carious material, instead of being inoculated into acid glucose broth, was plated on glucose agar at pH 5·6 and pH 7.

B. acidophilus was isolated from 7 teeth, three being in an advanced stage of caries, and the other 4 having shallow foci with considerable erosion of the enamel. The results of the previous experiments were, therefore, confirmed.

On the pH 7 plates an organism growing in peculiar dull, greyish-white colonies attracted attention. It was isolated with considerable frequency, especially from early cases, and occurred once in pure culture. On investigation it proved to be a streptococcus of very distinctive characteristics, and although not acidophile, an extremely active producer of acid. It has not, apparently, been previously described, and the name *Streptococcus mutans* is proposed for it.

The only organisms other than *B. acidophilus* and *S. mutans* isolated with any degree of frequency were the ordinary mouth streptococci, mainly of the *S. salivarius* type.

INCIDENCE OF *STREPTOCOCCUS MUTANS* IN CARIES.

As *S. mutans* appeared to be fairly constantly present in early carious lesions, and as it was found to be a powerful acid producer, 50 teeth in the earlier stages of decay were examined in order to ascertain the relative

frequency with which it could be isolated; 40 of these were cases of fissure caries, selected because there is frequently present in this variety of the disease a considerable area of decay in the dentine with little or no loss of enamel substance, and material more or less free from secondary infection and from contamination with surface bacteria could be obtained. In the other 10 the carious foci were on an approximal surface, being confined to the enamel in nine.

Technique of isolation.—In the fissure cases a modification of that previously used was adopted. The roots were cut off and the crown sawn from below; the carious material was drilled out with a fine drill, suspended in a drop of normal saline, and plated on glucose agar at pH 7. Figs. 1 and 2 are photographs of decalcified sections of two teeth, showing the invasion of the dentine by bacteria and the position from which the material for culture was taken; in a number of cases, however, it was not possible to obtain the material so deeply, as the lesions did not extend far enough into the dentine. Two of the approximal cases were treated in a similar manner, but the remainder were simply drilled from the outside. Plates were examined at the end of forty-eight hours, and, after colonies had been picked off, a general scraping was inoculated on glucose agar at pH 4 in order to isolate *B. acidophilus* if present.

Results.—*S. mutans* was isolated from 36 of the 50 teeth. Of the 40 fissure cases it was isolated from 27, being found in pure culture in 8; 4 plates were sterile, and on practically all the remainder colonies identical with those of the streptococcus were seen, although it could not be isolated. It is rather difficult at times to pick these colonies off the surface of the medium without their becoming contaminated from neighbouring ones, and almost impossible to separate *S. mutans* from a contamination, on account of the slowness of its growth and the rapidity with which it becomes overgrown. In 7 of the cases from which pure cultures were isolated the enamel was apparently intact and the material for cultivation obtained fairly deeply in the dentine, while in the eighth, although there was some erosion of the enamel, the material was obtained very deeply and at the extreme edge of the lesion. *S. mutans* was isolated from 9 of the approximal cases, and numerous colonies were seen on the tenth plate. Although, as might be expected, a large variety of organisms grew on these plates, *S. mutans* very markedly predominated in the great majority of cases, sometimes outnumbering all others in the ratio of 4 or 5 to 1.

B. acidophilus was present in 9 fissure and 5 approximal cases, all the former having foci of caries so shallow that the enamel, which was in most cases eroded, broke away with the drill and the surface of the tooth was encroached on in taking the specimen. In 11 of the cases from which *B. acidophilus* was isolated, *S. mutans* was also present.

Streptococci of various kinds, and more rarely staphylococci, were the only other organisms which appeared on the plates with any degree of regularity. There is little doubt that they were contaminations from the surface, as in cases from which the specimens were obtained deeply they were present in extremely small numbers, if at all. It is obviously not easy to sterilize the surface of a tooth thoroughly without allowing the heat to penetrate deeply enough to kill the bacteria in the carious focus.

STREPTOCOCCUS MUTANS.

Morphology.—When grown on a medium with a neutral or alkaline reaction, *S. mutans* is a non-capsulated coccus, about 0·75 μ in diameter, growing

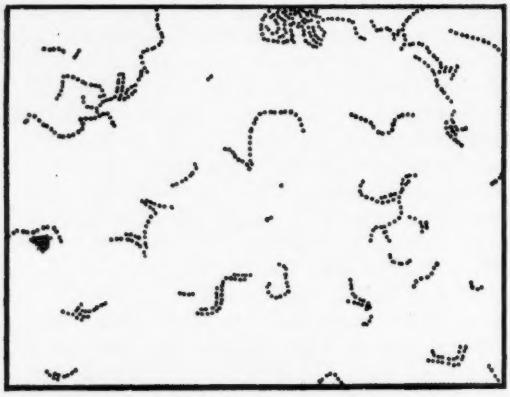

FIG. 3.—*S. mutans.* 24-hour broth culture. × 1200.

in chains of medium length: if, however, the medium becomes acid, it assumes the form of a bacillus. The length of the bacillary form varies from 1·5 μ to 3 μ or more, being greater on a solid than in a liquid medium. Figs. 3,

FIG. 4.—*S. mutans.* 24-hour glucose-broth culture. × 1200.

4 and 5 show the appearances of smears from 24-hour-old broth, glucose broth, and glucose agar cultures respectively.

Cultural characteristics.—It is aërobic and facultatively anaërobic. The optimum reaction for growth is about pH 7, none being obtained if the acidity of the medium is above pH 5·6. There is no growth at 22°C.

On glucose agar, small, dull, greyish-white, pin-point colonies, which seem to be imbedded in the medium, appear in about 24 hours. Seen under a low power of the microscope these colonies are opaque, brownish and round, with a lighter centre, a " hairy " texture, and a sharply-defined, usually slightly villous margin (Fig. 6). Where crowded together they remain minute, having little tendency to coalesce, and the growth has a matte appearance like ground-glass, but where more widely separated they attain a diameter of a millimetre or more in 3 or 4 days, become heaped up and irregular in outline, and are not unlike young colonies of *Actinomyces bovis* (Wolff and Israel). It is rather difficult to pick individual colonies off the surface of the medium, as they are very coherent and run along in front of the needle. Cultures on glucose agar survive for about 3 weeks. In glucose broth there is at first a general turbidity, which soon settles to the bottom as a granular deposit. Acid is very

FIG. 5.—*S. mutans*. 24-hour glucose-agar culture. × 1200.

rapidly produced, the medium, originally pH 7, giving a reaction of pH 4·2 in 24 hours; it never becomes more acid than this, however, and the organisms die out within the next 24 to 48 hours. On agar the growth resembles that on glucose agar, but is very feeble, while in broth there is a slight flocculent deposit with little or no turbidity. On Dorset's egg medium, serum media, and in fresh blood-serum a good growth is obtained, and cultures survive for a considerable time on these media as no acid is produced. On blood-agar the colonies are similar to those of other streptococci, and there is usually neither hæmolysis nor discoloration, although at times a very slight greenish colour has been observed after 48 hours. Gelatin is not liquefied.

Bio-chemical and serological reactions.—All the strains isolated ferment glucose, lactose, raffinose, mannite, inulin and salicin with production of acid; no gas; dulcite is not fermented.

An agglutinating serum was obtained by injecting a rabbit with increasing doses of a strain of *S. mutans*. This serum, which had a titre of 1 in 10,000 for its homologous strain, agglutinated all others in a dilution of 1 in 5000 or

higher; *S. salivarius*, *S. fæcalis*, *S. pyogenes* and *B. acidophilus* were not agglutinated even in a dilution of 1 in 40. Absorption tests were carried out with the homologous strain and 4 others chosen at random; the agglutinins were mutually absorbed. It may be presumed, therefore, that all strains of *S. mutans* are identical.

PRODUCTION OF ARTIFICIAL CARIES.

Three healthy teeth were sterilized in the autoclave and placed in glucose broth (pH 7) cultures of *S. mutans*. Access of the organisms to the pulp cavity was prevented by covering the roots with india-rubber teats, and the medium was changed daily so that the teeth might not remain for any length of time in an acid solution. The growth adhered markedly to the surface of the enamel, it being impossible to remove it completely by scraping or by washing under a moderately powerful stream of water. The teeth were allowed to remain in the cultures for 7, 9½ and 13 weeks respectively, and on removal they all showed considerable decalcification of the enamel, especially on the edges of the cusps and in the fissures. No naked-eye change was apparent in the dentine of the first tooth, although sections showed a commencing invasion by bacteria, but in the second and the third, brown areas were found at the bottoms of the fissures, and microscopic examination showed the dentinal tubules in these areas to be invaded by bacteria. Figs. 7 and 8 are photographs of decalcified sections of these teeth, while Figs. 9 and 10 demonstrate that the appearances seen in natural and artificial caries are practically identical, the greater dilatation of the tubules in the former being due to the fact that the decay was more advanced. In Figs. 9 and 10 the organisms seen are cocci, but the tubules in many cases appear to be filled with bacilli, both in the natural and artificial lesions.

A fourth tooth was placed in a culture of *B. acidophilus* (McIntosh, James and Lazarus-Barlow's type I), exactly the same conditions being observed. At the end of 13 weeks only a superficial decalcification of the enamel was found, and microscopic examination showed no invasion of the dentine.

It is proposed to undertake further experiments of this nature, the medium being changed automatically 5 or 6 times daily, so as to imitate mouth conditions more closely, and animal experiments are at present in progress.

SUMMARY.

B. acidophilus could be isolated only from teeth in which cavities had formed, or in which the foci of caries were shallow and contamination from the surface could not be excluded. This organism, although it will grow in a neutral or slightly alkaline medium, prefers an acid one, and would therefore find suitable pabulum in the carious cavities. *S. mutans*, on the other hand, grows best in a medium with a reaction approximating that of the saliva, is found in the earliest stages of decay, and can be isolated in pure culture from carious dentine which is more or less effectually protected from secondary infection or surface contamination.

The artificial caries experiments show that while *S. mutans* is able to

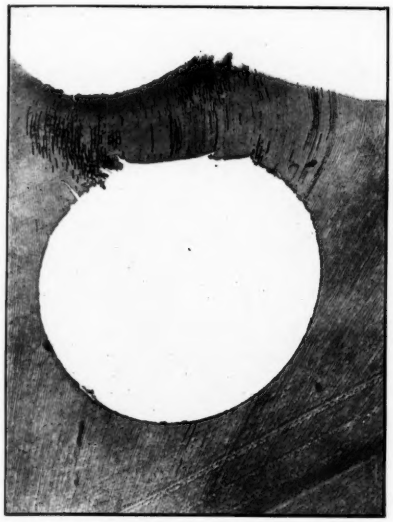

FIG. 1.
Decalcified area of carious dentine. The hole was made by the drill in taking the specimen for culture. *S. mutans* isolated. × 44.

FIG. 2.
As in Fig. 1.

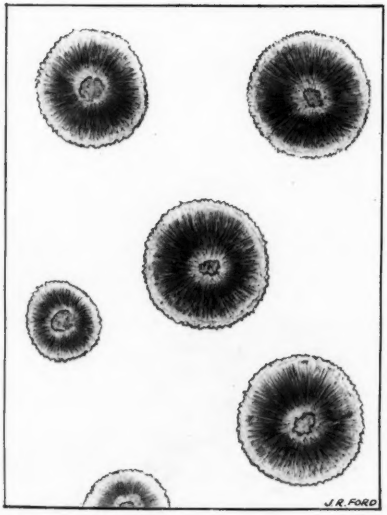

FIG. 6.
S. mutans. 24-hour colonies on glucose-agar.

FIG. 7.
Artificial caries. Decalcified section of tooth kept in cultures of *S. mutans* for 9½ weeks. × 70.

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FIG. 8.
As in Fig. 7. 13 weeks.

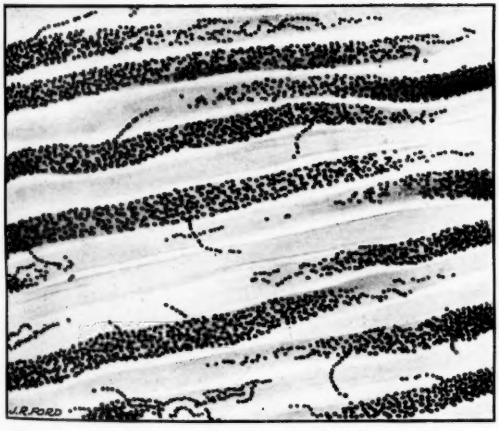

FIG. 9.
Natural caries. × 1200.

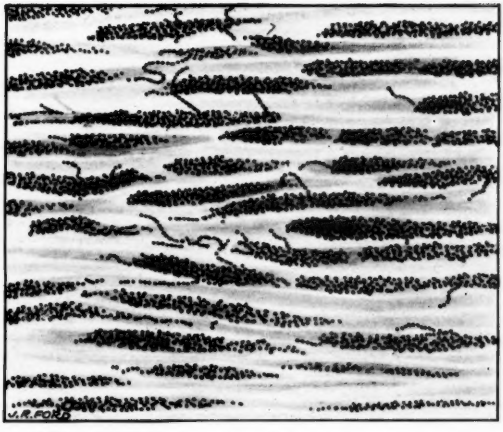

FIG. 10.
Artificial caries. Same specimen as Fig. 8. × 1200.

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FIG. 8.
As in Fig. 7. 13 weeks.

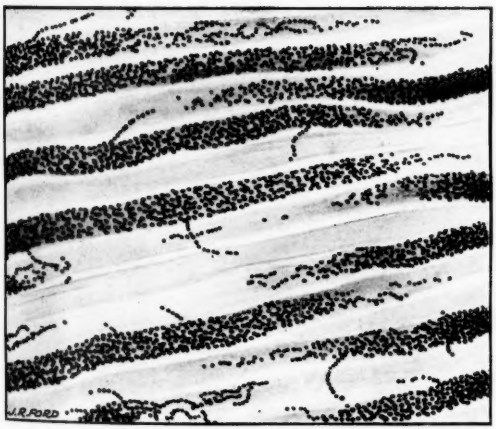

FIG. 9.
Natural caries. × 1200.

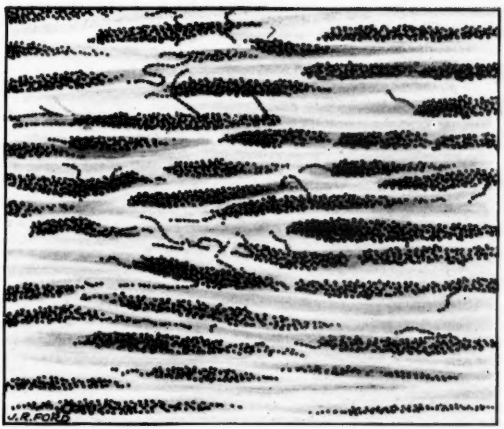

FIG. 10.
Artificial caries. Same specimen as Fig. 8. × 1200.

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decalcify the enamel and penetrate into the dentine when the conditions which obtain in the mouth are to a certain extent approximated, *B. acidophilus* produces only a superficial decalcification of the enamel, which can be accounted for by the acidity of the medium. In McIntosh, James and Lazarus-Barlow's experiments the medium was changed once a week, and the teeth therefore remained in a concentration of acid sufficiently high to produce decalcification of the enamel for fully 6 out of every 7 days. The fact that the colonies of *S. mutans* adhere closely to the surface of the teeth appears to be of great importance, as a local concentration of acid in contact with the enamel is thereby produced, and this could obviously give rise to rapid decalcification independently of the acidity of the medium as a whole.

The streptococci other than *S. mutans* may be disregarded, as they are comparatively feeble acid producers, and were either entirely absent, or present only in small numbers in cases where the carious dentine was reasonably protected from secondary infection or outside contamination.

The evidence suggests that caries is due to infection of the teeth, in certain circumstances not yet made clear, by a hitherto undescribed streptococcus, *S. mutans*. Whether the primary infection is facilitated by defective structure of the teeth, such as Mrs. Mellanby (1918, 1923) has been able to produce in puppies by restricted diets, remains to be proved. Experiments in this direction are in progress.

My thanks are due to Sir Almroth Wright for extending to me the hospitality of his laboratories, to Dr. Leonard Colebrook for valuable suggestions and helpful criticism, to Messrs. Norman G. Bennett, W. H. Dolamore, A. Templar Barritt and Robert McKay for clinical information and assistance in the collection of material, to Mr. J. E. Barnard for the microphotographs, to Dr. V. D. Allison for carrying out the animal inoculation, and to Dr. P. Lazarus-Barlow for cultures of *B. acidophilus odontolyticus*.

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ON THE ACCURACY OF MEASUREMENT OF SMALL VOLUMES OF FLUID WITH A CAPILLARY PIPETTE.

INCORPORATING A DESCRIPTION OF A GRADUATED PIPETTE FOR RAPIDLY AND ACCURATELY MAKING A SERIES OF DILUTIONS OF A FLUID.

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THE question of the accuracy with which small volumes of fluid can be measured with capillary tubes is a very important one, as many investigations on human blood can only be done in small volumes, and unless a reasonable degree of accuracy can be attained grave doubts would have to be cast on the results of many methods. This question is of importance, especially to the followers of Sir Almroth Wright, who have in the past been in the habit of using small quantities of fluid and capillary measuring tubes for most of their routine and research operations. The work about to be described was the result of an article by Sir Frederick Andrewes (1919), in which he alluded to certain considerable errors which seemed to creep into measurements with capillary pipettes, and it seemed necessary to demonstrate if possible that such methods were accurate within the limits desired.

In measuring volumes of fluid with capillary pipettes, just as with pipettes of larger calibre, there are several errors which may arise, and it is possible that some of these are magnified by the small volume of the pipette. In the first place there is the question as to whether with ordinary skill fluid can be accurately taken up to a mark made on a pipette with a grease-pencil. (Obviously here the nature of the mark is of importance, and in all future discussion I am presuming that the mark is as fine as is consistent with its ready visibility.) In the second place there has to be considered the amount of fluid which adheres to the inner wall of the pipette after the volume has been discharged, and lastly there is the fluid which adheres to the outside of the pipette around its tip. These possible errors will be discussed and separated as far as possible.

Before going on to describe the experiments it would be well to indicate briefly the method of calibrating the pipettes employed. This was in all cases the method described by Sir Almroth Wright (1912). By means of a standard "automatic" pipette a measured volume of clean mercury was introduced into a capillary pipette, run down to the end of this, and a mark made on the pipette such that the capacity of the tube from the mark to the tip exactly corresponded with the volume of the mercury. If in such a pipette a fluid which does not wet the walls of the tube is taken up to the mark and delivered, the amount delivered will exactly correspond with the volume marked on the

pipette (presuming that the fluid has been accurately measured). If, however, the fluid wets the glass, the amount delivered will be the volume marked on the tube minus the amount which remains adherent to the walls. This amount of fluid which remains in the tube after the volume has been discharged has been conveniently termed by Sir Almroth Wright the " wash," and as such it will be referred to in this discussion. All watery fluids will of course wet the wall of a glass pipette, but if the wall be coated with paraffin, then there will be no tendency for the fluid to adhere to the pipette and the error of the wash will be eliminated.

We can therefore have pipettes coated with paraffin inside and out in which the error of the wash and the error due to fluid adhering to the outside of the pipette are done away with, others coated only on the outside in which the only error (except that of incorrect measurement) is that of the wash, and pipettes without any paraffin coating with which all the errors may occur.

The accuracy of the amount delivered by a paraffined pipette and the extent of the " wash " can to some extent be seen in the following preliminary experiments:

Two capillary pipettes were taken, one coated with paraffin and the other left bare, and on each a mark was placed corresponding to a volume of 250 c.mm.

The length of tube occupied by this volume measured 162 and 220 mm. in the paraffined and the non-paraffined pipettes respectively. With the paraffined pipette two volumes of water were measured and placed on a slide coated with paraffin. The first of these drops was then taken up with the other pipette, and it was found that the fluid came exactly to the 250 c.mm. graduation mark. The fluid was then expelled from the non-paraffined pipette at a moderate rate and the second of the measured volumes was taken up. This time the fluid came to a level higher than the graduation mark and it was found that there was an increase of 3·1 per cent., this increase being due to the fluid which had remained in the non-paraffined pipette after the first volume had been expelled.

The experiment was then done in the reverse way. With the non-paraffined pipette volumes of 250 c.mm. were taken up and delivered on to a paraffined slide from which they were taken up into the paraffined graduated pipette. Three volumes measured in this way were found to give a deficiency of 3·3 per cent.

With the same two pipettes volumes of strong caustic soda were delivered into test-tubes, and these volumes were titrated with weak sulphuric acid. The volume delivered from the paraffined pipette required 40·7 c.c. of acid to neutralize it, whereas the other required only 39·2 c.c., making a difference of 3·6 per cent.

In these preliminary experiments it will be seen that whatever was the method adopted of measuring the volume delivered, the amount of the "wash" was about the same—3·1 to 3·6 per cent. In these experiments, however, the volume used was a large one (250 c.mm.). In the next experiment volumes of 10 c.mm. were employed, and to avoid any error due to fluid adhering to the outside of the pipette it was coated outside with paraffin. With the pipette used the length of the 10 c.mm. volume was 34 mm.

Ten volumes of normal NaOH were measured out with the pipette. Five of these volumes were simply blown out into test-tubes which contained a little neutral water, while with the other five volumes the inside of the tube was washed out by taking up water and blowing it out several times. (Between each measurement the pipette was, of course, washed and dried.)

In the first five volumes the amount delivered therefore was 10 c.mm. minus the wash which adhered to the inner walls of the capillary tube, while in the second five volumes the whole ten c.mm. was delivered. These volumes of NaOH were then titrated with weak HCl solution, and as the amount of fluid was so small it was necessary to use a dropping method in the titration. The results obtained were as follows (they have to be divided into two groups as two different acid solutions were used in the titrations) :

	Volume minus wash.	Volume plus wash.
Group 1 . .	194 drops .	202 drops
	196 ,, .	202 ,,
	196 ,, .	202 ,,
Group 2 . .	200 ,, .	206 ,,
	200 ,, .	206 ,,
Mean . .	197·2 ,, .	203·6 ,,

The difference due to the wash in this experiment amounts to 3·1 per cent., which corresponds closely with the figure obtained for the larger volume in the previous experiment.

THE ACCURACY WITH WHICH A VOLUME OF TEN CUBIC MILLIMETRES CAN BE DELIVERED.

This is a volume which is a very average one for the user of capillary methods to measure. The previous preliminary experiment showed that considerable accuracy could be obtained, but a further and more exhaustive series of tests were made. Three sets of observations were made—the first in which the pipette was paraffined both outside and inside, the second in which the pipette was paraffined on the outside only, and the third in which the pipette was not paraffined.

In each case the fluid (water) was taken up to the graduation mark on the pipette and then expelled at a moderate rate on to a paraffin-covered slide. The volume was measured by taking up the drop of water which had been expelled on to the paraffin slide in a very fine capillary pipette which had a mark corresponding to a 10 c.mm. volume. The height of the fluid above or below this mark was measured so that the exact amount of the fluid could be very accurately estimated.

A consideration of Table I will show us the amount of the various errors to which such measurements are subject. With the pipette paraffined inside and outside the wash was eliminated, and what was really being estimated was the accuracy with which fluid could be drawn up to a mark on a capillary pipette. Of seven estimations four were absolutely accurate, and the maximum error amounted to 0·85 per cent.

With the pipette paraffined only on the outside we have a measure of the wash which adheres to the lumen of the tube after the volume is expelled.

The deficiency in this series of observations varied from 1·98 to 2·6 per cent.; the mean deficiency was 2·2 per cent., which may be accepted as the amount of the wash.

Deducting this wash from the deficiency found by experiment in order to ascertain the accuracy of working, we find that the mean error of working is 0·16 per cent. and the maximum error of seven estimations is 0·38 per cent.

TABLE I.—*Accuracy attained in measuring 10 c.mm. Volumes with a Capillary Pipette.*

Type of pipette used.	Length of 10 c.mm. volume on pipette.	Error per cent.	Mean deficiency per cent.
Paraffined inside and outside	87 mm.	0	
		0	
		0	
		0	0
		+ 0·48	
		− 0·85	
		+ 0·51	
Paraffined on the outside only	62 mm.	− 2·41	
		− 2·22	
		− 2·01	
		− 2·60	2·2
		− 2·17	
		− 1·98	
		− 2·16	
Not paraffined	84 mm.	− 1·22	
		− 1·97	
	71 mm.	− 2·17	
		− 1·40	2·04
		− 2·74	
	72·5 mm.	− 2·70	
		− 2·10	

With the third series in which the pipette was not paraffined we have the error of the wash, and also that due to any fluid which might adhere to the outside of the tube. It will be seen that in this case in seven estimations there was a mean deficiency of 2·04 per cent., which may be taken as the wash, and if this be added to each result, we find that there is a mean error of working of 0·43 per cent. and a maximum error of 0·82 per cent. There is therefore an appreciably higher working error than when the pipette is paraffined outside, and this is probably due to an uncertain quantity of fluid adhering to the outer wall of the tube. The technique employed of blowing out the volume on to a paraffined slide tended to exaggerate this error, as there was more tendency for the fluid to adhere to the pipette than to the slide. It was thought that if the fluid had been blown out into water the irregularity would have been less, so nine volumes of .5 c.mm. of strong caustic potash solution were measured in a non-paraffined pipette and blown out into test-tubes containing a little neutral water, care being taken that when the fluid

was being blown out the tip of the pipette was just below the surface of the water. The contents of the test-tubes were then titrated with weak sulphuric acid by the drop method with the following results:

5 tubes required 75 drops for neutralization.
2 „ „ 74 „ „
2 „ „ 76 „ „

This last experiment shows that even with so small a volume as 5 c.mm. fairly accurate measurements may be made with a simple capillary pipette.

If we sum up the results of the observations tabulated in Table I, we must come to the conclusion that 10 c.mm. volumes can be measured with a capillary pipette with an accuracy more than sufficiently delicate for the adequate performance of the serum and blood tests. In making these observations a rather slender pipette was used, and the emptying of the pipette on to the paraffined slide was done more slowly than is customary in ordinary work. The amount of the wash therefore amounted to only about 2 per cent., as against somewhere about 3 per cent. as is usual when the pipette is emptied at the ordinary working rate. It was necessary to empty the pipette slowly using this technique, otherwise there was a risk of the last portion of fluid rushing out too quickly with consequent bubbling, running of the fluid back up the pipette, etc. When this occurred it was found that the deficiency was much greater than that shown in Table I, and in three instances where this happened measurements were made and it was found that the fluid was deficient by between 4 and 5 per cent. Such volumes therefore as are delivered with much bubbling should, if accuracy be required, be discarded.

VARIATION OF THE AMOUNT OF THE WASH WITH THE CALIBRE OF THE TUBE AND WITH THE RAPIDITY OF THE RATE OF EXPULSION OF THE FLUID.

It seems obvious that the amount of fluid which adhered to the walls of a pipette would be greater as the calibre of the pipette became smaller. In ordinary working, however, the different calibres of pipettes used for the measurement of small quantities only vary by three or four times in their calibre, and as a result of repeated experiment with narrow and wide pipettes, it has been found that where the rate of expulsion of the fluid is approximately constant there is practically no difference between the wash left in the narrow and in the wide pipette.

It is common knowledge to all workers with capillary pipettes that more fluid remains behind, in other words, the wash is larger, when the fluid is expelled rapidly than when it is expelled slowly. The wash left in a fine bore capillary pipette (80 c.mm. occupied a length of 17·9 centimetres) was estimated after volumes of 80 c.mm. had been blown out from the tube (*a*) very rapidly, (*b*) at ordinary working pace, and (*c*) very slowly. The results obtained were as follows:

	Mean deficiency (wash) of three estimations.
When delivery was very rapid	8·85 per cent.
When delivery was moderately rapid (ordinary working pace)	3·0 „
When delivery was very slow	0·77 „

These figures probably represent the extremes which are met with using such pipettes, and in ordinary working such variations could not occur without gross negligence of the elementary precautions which should be taken. After some practice with a teat and a capillary pipette the worker acquires a certain amount of constancy in the rapidity of his working, and, as has been shown in previous experiments, small variations in the rate of expulsion can have little bearing on the results.

THE ERROR INVOLVED IN MAKING A GRADUATED SERIES OF DILUTIONS OF A FLUID.

In the preceding experiments it has been shown that it is possible with a fair degree of accuracy to deliver a small volume from a capillary pipette. It has been shown also that at the ordinary rate of working, the wash—that is the amount of fluid remaining in the tube after delivery of the volume—amounts to about 3 per cent. of the volume. Further, it has been shown that if the pipette is coated inside with paraffin the wash is done away with and the full volume is delivered.

In the routine of bacteriological work it is frequently necessary to make a series of twofold dilutions of fluids, and the question of the accuracy of such series of dilutions was investigated by Sir Frederick Andrewes, who found that, using the utmost care, there was a progressive error in the direction of making the dilution too strong, such that at the eighth dilution the error amounted to about 15 per cent.

If consideration be given to the method of making these dilutions, and if the errors which have been demonstrated to be inherent in the method be taken into account, we should be able to arrive theoretically at the error of the method, presuming it to be performed in a skilled manner.

In the diluting method most commonly used a mark is made on a capillary pipette some 2 or 3 centimetres from the end denoting a volume measured or arbitrary. With this pipette a series of volumes of the diluting fluid are placed on a slide, and to the first of these is added with the same pipette one volume of the fluid to be diluted (after washing out the pipette with this fluid). Mixing is carried out by drawing the fluid up and down the pipette several times (care being taken that the fluid does not pass up the pipette much higher than the graduation mark), and then with the same pipette a volume of the first dilution is carried over to the second drop of the diluent, mixed, and so on till the series is completed.

It has been shown in the previous experiments that the wash amounts to about 3 per cent. of the total volume, so that each of the volumes of diluent would be 3 per cent. short of the capacity of the pipette up to the mark. The volume of the fluid to be diluted would when first expelled be short by the same amount, but in the process of mixing the whole of the wash would be incorporated. Thus, supposing the original volume marked on the pipette to be 100 c.mm., this volume of the fluid to be diluted would be mixed with 97 c.mm. of the diluent, giving an error in the first dilution of $1\frac{1}{2}$ per cent., and this error would be progressive, so that at the eighth dilution it would amount to about 13 per cent. It will be seen therefore that the "wash"

could account for practically the whole of the error found by Sir Frederick Andrewes in his dilutions.

There is, of course, with the unparaffined pipette a small error due to the fluid adhering to the outside of the pipette, but if care is taken not to dip the pipette deep into the fluid it is only the tip of the pipette which will be wetted and the error will be small. The error due to inaccurate measurements of the fluid should not exist for practical purposes. It has been shown in the experiments quoted above that it need only be very small, and as it occurs on both sides of the normal these small errors would, in a series, tend to neutralize themselves.

QUESTION AS TO WHETHER AN ACCURATE SERIES OF DILUTIONS CAN BE MADE USING ONLY MINUTE QUANTITIES OF FLUID.

An experiment was made to see whether accurate dilutions of small quantities of a fluid could be made when precautions were taken to prevent the errors which have been discussed above. It might be thought that by the use of a paraffined pipette all these errors might be eliminated, but it is found that when it is desired to make dilutions of a fluid, such as serum with a paraffined pipette, there are very definite technical difficulties. Although it is possible with great accuracy to deliver a series of volumes of water or salt solution with a paraffined pipette, it will be found that when a series of dilutions of serum have to be mixed with such a pipette there is apt to be a great deal of bubbling, rendering the measurement of the volume a tedious and uncertain procedure. For this experiment, therefore, where the greatest possible accuracy was desired, two pipettes were employed. These were narrow capillary pipettes similar to those which have in the past been used largely for the estimation of the opsonic index. One of these was paraffined inside and outside and the other was paraffined only on the outside. The former will be referred to as pipette 1 and the latter as pipette 2. With the same globule of mercury of exactly 40 c.mm. volume the two pipettes were calibrated, so that the capacity of the capillary from the tip to the mark was exactly 40 c.mm. The length of tube occupied by this volume was 13 centimetres in pipette 1 and 11 centimetres in pipette 2. With pipette 1 eight volumes of 40 c.mm. of neutral distilled water were measured out in separate drops on a glass plate coated with paraffin. (It has been shown above that with such a paraffined pipette the exact quantity is delivered.) With pipette 2 a series of twofold dilutions of a strong solution of KOH were made in the manner described above. Care was taken in making the dilutions not to cause bubbling of the fluid or to take the fluid during the process of mixing higher than the graduation mark. When the dilutions were completed the drops of fluid on the paraffined slide were transferred to test-tubes containing about 5 c.c. of neutral distilled water to which had been added some phenol red. They were then titrated with N/250 HCl. With the first four dilutions titrations could readily be made with a burette, but with the weaker dilutions the drop method had to be employed, precautions being taken to ensure the size of the drops being equal. The results obtained were as follows:

Dilution.	Amount of acid required to neutralize.	Amount of acid reduced to terms of c.c.	Calculated result.*	Percentage error.
Undiluted	29·8 c.c.	29·8	—	—
1 in 2	15·0 ,,	15·0	14·9 c.c.	0·67 +
1 in 4	7·5 ,,	7·5	7·45 ,,	0·67 +
1 in 8	3·8 ,,	3·8	3·725 ,,	2·00 +
1 in 16	1·85 ,,	1·85	1·8625 ,,	0·78 —
1 in 32	107 drops†	0·922	0·9321 ,,	1·00 —
1 in 64	53 ,,	0·457	0·4656 ,,	1·9 —
1 in 128	27 ,,	0·233	0·2328 ,,	0·01 —
1 in 256	13 ,,	0·112	0·1164 ,,	4 —
1 in 512 (2 volumes.)	13 ,,	0·056	0·0582 ,,	4 —

This experiment shows that with small volumes and a fine capillary tube a series of dilutions can be made which rivals in accuracy (indeed, it is even more accurate) the series of dilutions made by a skilled chemist quoted by Sir Frederick Andrewes.

The errors vary on both sides of the calculated quantity, and are probably due as much to the error of titration as to the error of dilution.

A SIMPLE AND RAPID METHOD OF MAKING A SERIES OF DILUTIONS WITH A CAPILLARY PIPETTE WHEREBY THE ERROR OF THE WASH IS ELIMINATED.

It is here proposed to describe a method of making serial dilutions which gives great accuracy, and at the same time is more rapid than the ordinary method of making serial dilutions. The method, however, requires rather more familiarity with the technique of the teat and the capillary tube than does the ordinary method described in the last experiment. It is necessary first to construct a pipette graduated in a special manner. The graduation may be by any particular scale which it may be desired to employ in any particular investigation, but for purposes of clarity it is necessary to choose one scale for the description of the construction of the pipette, and the one chosen will be a pipette suitable for a series of agglutination or similar tests commencing with a 1 in 2 dilution and finishing with a 1 in 200 dilution.

A piece of glass tubing of about 6 mm. bore is drawn out in such a way that a tapering tube is produced ending in a comparatively fine capillary. Into this tube a volume of 2·5 c.mm. of mercury is introduced and run down into the fine portion of the capillary, when marks are made with a fine-pointed grease-pencil corresponding with the limits of the mercury volume. The mercury is now run back and to it is added a volume of 5 c.mm. The whole 7·5 c.mm. is now run down till its lower limit reaches the lower mark and another mark is made at the upper limit. The volume then between the second and third marks will be 5 c.mm. By the same procedure volumes of 10, 20, 50, 100, 250 and 500 c.mm. are added and marked on the stem of the pipette, and when the calibration has been completed the pipette is cut off at

* The calculations are based on the assumption that the titration of the first (undiluted) volume is accurate.

† The number of drops to 1 c.c. was estimated and it was found to be 116.

the lowest mark, and if necessary a throttle is introduced to increase the ease and accuracy of working. Such a pipette is illustrated in Fig. 1.

The method of using the pipette is as follows:

A series of eight small test-tubes are taken and arranged in a stand. Into each of these tubes, with the exception of the left-hand one, is placed 0·5 c.c. of normal salt solution (or other diluent). By means of the pipette described there is removed from the tubes, commencing at the second from the right, volumes of 2·5, 5, 10, 20, 50, 100 and 250 c.mm. (These volumes are discarded.) The same pipette (for extreme accuracy the pipette should be first washed and dried) is now filled to the top mark with the fluid to be diluted, which for descriptive purposes may be said to be serum, and volumes of this are delivered into the tubes in order commencing with the left-hand tube.

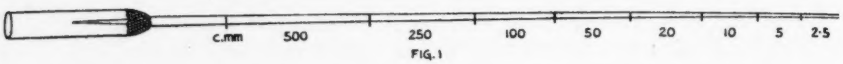

FIG. 1

The composition of the fluid in the various tubes will then be as set forth below.

	Tube No. (from left to right).									
	1.	2.	3.	4.	5.	6.	7.	8.	9.	
Salt solution .	0	250	400	450	480	490	495	497·5	500	c.mm.
Serum .	500	250	100	50	20	10	5	2·5	0	,,
Total volume .	500	500	500	500	500	500	500	500	500	c.mm.

It will be seen that as a result there is the same volume of fluid in all the tubes, while there is a progressive series of dilutions of the serum from 1 in 1 to 1 in 200. If we imagine this to be an agglutination test, all that has to be done now is to add to each tube 0·5 c.c. of the bacterial suspension, shake the tubes, and incubate them.

The actual amount delivered will not, however, in any one instance be the amount set forth above, as in every operation there will be left in the measuring pipette a wash of the fluid, so that the amount delivered will be the amount as indicated by the graduation of the pipette minus the wash, which we can take to be at the ordinary rate of working to be about 3 per cent. The fact that an error of the same magnitude occurs *in the same direction*, both in the diluent and in the fluid to be diluted, can be neglected as the two errors cancel one another, and the amount of the dilution should be the same as if the whole volume had been delivered.

Pipettes of this type can be used for almost all the bacteriological tests which involve serial dilutions. The author originally used them for the titration of complement, and for this purpose pipettes were used in which the volumes increased by simple arithmetical progression, namely, 10, 20, 30, 40, 50 and 60 c.mm. (or where the progression was by 5 c.mm. instead of by 10, the limits being the same). It will be seen that with such a pipette, if the diluent is first taken up and distributed in volumes along a series of tubules from *right to left*, and then the fluid to be diluted is taken up (after washing

out the pipette with this fluid) and distributed in a similar manner from *left to right*, in each operation leaving one tube at the end of the series untouched, the series of dilutions will be complete and the volume in each of the tubules will be the same. When, however, the progression is other than a simple arithmetical one, it is necessary to use the method described above or to construct a complementary pipette of the same type, but on which the measured volumes are such that when added to these on the prepared delivery pipette they will be equal. An example of such a pair of complementary pipettes is shown in Fig 2. On the pipette with which the fluid to be diluted is to be delivered the volumes marked off are 2·5, 5, 10, 20, 40 and 80 c.mm., while on the complementary pipette which would be used for the diluting fluid the volumes are 40, 60, 70, 75, 77·5 and 80 c.mm. Then, supposing that serum was to be diluted with salt solution, there would result after one

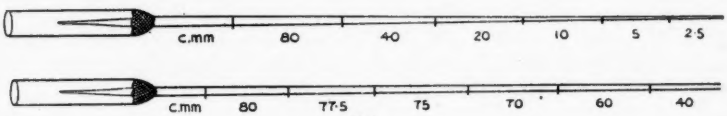

FIG. 2.

operation of filling and emptying of each of these pipettes along a series of tubules in the manner described the following mixtures:

	Tube No. 1.	2.	3.	4.	5.	6.	7.	
Salt solution	0	40	60	70	75	77·5	80	c.mm.
Serum	80	40	20	10	5	2·5	0	,,
Total volume	80	80	80	80	80	80	80	c.mm.

A set of such pipettes made in one's spare time to suit the various tests which require to be done saves much time in the long run, and if reasonable care is taken these capillary pipettes, although they appear to be very fragile, will last for long periods without breaking. The first pipette of this type which the author made about twelve years ago is still in use, although many hundreds of complement titrations have been done with it during that period.

With the use of such pipettes the error of the wash is eliminated, and the accuracy of the dilutions will depend almost entirely on the ability of the worker to deliver volumes accurately to a mark on the pipette. When such skill has been acquired, then the various series of dilutions can be made more accurately and with much less trouble than by any other pipette method in use.

SUMMARY.

It has been shown that measurements of 5 and 10 cubic millimetres can be made with ordinary capillary pipettes with more than sufficient accuracy for use in the ordinary serological work of a bacteriological laboratory.

The wash—that is, the amount of fluid which adheres to the inner wall of

the pipette—amounts to, at the ordinary rate of working, about 3 per cent. of the volume measured. In making a series of dilutions in the ordinary way, the fact that a wash of about 3 per cent. remains in the tube introduces a progressively increasing error of about 1·5 per cent. with each dilution, so that at the eighth dilution the theoretical error would be about 13 per cent.

A pipette is described whereby series of dilutions can be made rapidly, and in which the error due to the wash can be eliminated.

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THE DEMONSTRATION BY THE FIXATION ABSCESS OF THE INFLUENCE OF SILICA IN DETERMINING *B. TUBERCULOSIS* INFECTIONS.

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In ordinary clinical terminology "phthisis" means pulmonary tuberculosis. The term "miner's phthisis," however, does not mean pulmonary tuberculosis occurring in miners, but is loosely used to indicate both silicosis, a form of industrial pneumonokoniosis, and silicosis associated with tuberculosis. Thus, according to Mavrogordato (1922), one of the questions arising in connection with miner's phthisis is, "What is the part played by pulmonary tuberculosis in the development of miner's phthisis?" and one of the conclusions arrived at by him is, "All the early stages of miner's phthisis *can* be produced by silica alone, without association with *B. tuberculosis* or other pathogenic organisms."

In the study of miner's phthisis, therefore, we may be, and usually are, confronted with two distinct pathological processes brought about by (1) a dust—silica; and (2) an organism—the tubercle bacillus.

Inasmuch as there is abundant evidence that the silicotic lung is much more vulnerable to the tubercle bacillus than any other form of pneumonokoniosis (Collis, 1915), it is clear that the second pathological process is a

* The expenses of this work were defrayed by a grant from the Medical Research Council.

complex one. It is, indeed, of fundamental importance, for it illustrates an aspect of infection to which attention has been directed in respect to anaërobic bacilli (Bullock and Cramer, 1919), namely the modification of the reaction between the host and the organism by some third extraneous factor.

The main pathological change in the silicotic lung is an extremely dense and irregular fibrosis brought about by the chemical rather than the physical or mechanical action of the silica. Exactly why such a lung should be especially vulnerable to the attack of the tubercle bacillus is unknown. Fibrous tissue is not usually more prone to bacterial invasion than highly organized tissues; in fact, if it were not for the protective peripheral fibrosis, the lesions of chronic tuberculosis would be even more dangerous than they are. And there is no actual evidence to show that the disorganization of the lymphatic drainage of the lung, which must follow to some extent on the fibrosis and obliteration of lymph-channels, is sufficient to render the tissues relatively incapable of resisting bacterial invasion.

It has been shown (Gye and Kettle, 1922) that if tubercle bacilli are injected along with silica into the subcutaneous tissues of the mouse, the bacilli proliferate readily in the area of coagulation necrosis which the silica causes, protected for some days from the cellular defences of the body. Although these observations can only have a very indirect bearing on the problem of miner's phthisis, they suggest that the presence of a definite cell poison in the silicotic lung must diminish the power of the tissue reaction, and so favour the progressive multiplication of the infecting bacilli. It is obvious that the matter requires further investigation, and the experiments described in this paper were undertaken with the object of solving certain questions arising out of the previous work.

DISCUSSION OF METHODS USED.

The proliferation of the bacilli in the local tubercle-silica lesion may be due to various causes:

1. To the mechanical protection of the bacilli by the coagulum from the defensive mechanism of the body.
2. To the rich pabulum furnished by the disintegrated cells.
3. To the stimulating action of the silica on the growth of the bacilli.

In the first two possibilities the silica would act merely as a suitable agent for bringing about tissue destruction, but in the third its action would be much more specific—on the bacillus rather than on the tissues.

The method of inoculating tubercle bacilli into the tissues, together with various substances designed to produce necrosis, is a crude one, and is also open to the objection that the dose of viable bacilli may vary in different animals. But if a condition of artificial septicæmia is brought about by inoculating the organism directly into the circulation, all parts of the animal's body, except the lungs, which, in the intravenous inoculations, are exposed first and most heavily to the infection, must receive a uniform dose, corresponding to their vascularity. By this method it is possible to study the influence of various kinds of trauma and other factors upon the localization and proliferation of tubercle bacilli, or of any other organism. Experiments

on these lines have yielded interesting results, but the method has limitations which must be appreciated in interpreting the findings. In attempting to modify experimentally the reactions of the tissues to *B. tuberculosis*, or to any other pathogenic organism, it is scarcely possible to obtain clear-cut, absolute results; the changes brought about can only differ in degree from those in control animals. If the resistance of the tissues and the pathogenicity of the organism are so nicely balanced as they are when the mouse is the host and the tubercle bacillus is the infecting organism, no agent can be expected to bring about a permanent modification of the infection, unless its influence is exercised more or less continuously for a considerable period. The tubercle bacillus lives practically symbiotically in the cell tissues of the mouse. Any agent which favours its growth must be present persistently, for if not, though a temporary hyperproliferation of the bacilli is brought about, this is not continuous; a small local bacterial population is converted into a large one, but further proliferation ceases if the agent is not present to induce it. The organisms eventually die, for they are not pathogenic, as in the rabbit or guinea-pig, and give rise to no progressive tissue lesions.

In the guinea-pig, on the other hand, human tubercle bacilli call forth progressive reactions, and the favouring agent only alters the process appreciably if lesions are examined in the early stages. Later, the ordinary progressive tuberculosis entirely obscures the process. Intermediate is the rabbit treated with human tubercle bacilli, which tend to produce in this animal a retrogressing lesion. Here, again, the lesions must be examined at a favourable stage of their development, or else, in the absence of the continuous action of the agent, the defensive mechanism of the animal comes into play, and bacillary growth is hindered, if not entirely prevented.

EXPERIMENTAL DATA.

Observations on Mice.

Experiment 7A.—Twenty adult mice were inoculated in the right flank with 5 mgm. of SiO_2 suspended in normal saline, and into the left flank with 5 mgm. of $CaCl_2$ in normal saline. The following day they received intravenously $\frac{1}{8}$ to $\frac{1}{2}$ c.c. of a moderately fine emulsion of tubercle bacilli (human). The mice were killed at periods ranging from 6 to 21 days after infection. In some animals the local lesions were unsatisfactory, or death occurred during the night, and decomposition rendered the tissues unsuitable for examination; but 13 mice provided suitable material. The calcium and silica lesions were carefully dissected out and microscoped, and the degree of tuberculization of the animals themselves was determined by microscopic examination of the lungs, livers, spleens, kidneys, and in a few cases the bone-marrow.

The lungs invariably contain enormous numbers of bacilli phagocytosed in large macrophages, which in some areas completely fill the alveoli. The livers, spleens and bone-marrows contain numerous small foci with moderate numbers of bacilli; renal lesions are extremely rare. The calcium lesions (Fig. 1) are all much larger than the silica ones—indeed, in half the animals they ulcerated and became secondarily infected, the infection in some cases

passing across the middle line of the body to involve the silica focus. They heal by the formation of much highly vascular scar-tissue and the muscles of the abdominal wall are frequently affected. The silica lesions (Figs. 2 and 3), on the other hand, are all localized, consisting of necrotic foci surrounded by fibrous tissue with characteristic macrophage reaction. The difference in bacterial content of the two lesions is, however, remarkable. In the silica focus enormous numbers of tubercle bacilli proliferate in the central necrotic zone, while in small peripheral foci and in the track of the needle where healing is taking place, very many more bacilli are present than in any other part of the body except the lung. An interesting feature of the reaction is that the primary area of necrosis induced by the silica persists for a very long time, and the active repair which is characteristic of the uncomplicated silica abscess is delayed and diminished in intensity. It would seem that the bacilli are present in such enormous numbers as to disorganize the defensive processes of the tissues.

Turning to the calcium lesions, although the tissue destruction is much more extensive than in the silica ones, and quite as intense, the number of tubercle bacilli is incomparably less. They never proliferate in masses in the necrotic foci, but are limited to small areas scattered through the lesion, where they may be free or phagocytosed. These areas are never large, nor are there more than a few in any individual lesion, so although here and there the bacilli occur in considerable masses, there can be no comparison between the degree of localized infection induced by the silica on the one hand and calcium on the other.

Experiment 9A.—Fifteen mice were inoculated in the right flank with 5 mgm. of SiO_2, and in the left flank with 2 mgm. of $CaCl_2$ together with a small quantity of carbon in the form of waterproof ink. They were killed at periods ranging from 25 to 90 days. Material suitable for examination was obtained from 9 animals.

The general findings are much the same as in Experiment 7A, but in the later stages there are comparatively few bacilli in the silica lesions, and no histological reaction which can be recognized as tuberculosis. In these "late" animals, too, the bacilli are disappearing from the lung, while in the liver and spleen they are very sparsely distributed. The calcium lesion is considerably modified by the presence of the carbon. The fine particles become aggregated together to form irregular masses of unequal size. These are grouped in a necrotic matrix forming a nodule which is enclosed by dense fibrous tissue. Disintegrating wandering cells are sometimes present in the nodules in considerable numbers, but the lesions are quite localized and there is no diffuse reaction. For the most part tubercle bacilli are completely absent from these lesions, but in one mouse killed 27 days after infection, a considerable number are present in the necrotic tissue of the lesion, occurring in small groups. They are not so numerous as in the average silica lesion, and are entirely absent from the tissues outside the limiting fibrous capsule. In this particular animal the main silica lesion was unfortunately not included in the portion of connective tissue examined, so that the relative distribution of bacilli in the two foci cannot be determined, but many organisms are present in cellular areas in the adjacent connective tissues.

Observations on Rabbits.

Similar experiments were carried out on a series of rabbits with similar results. Inasmuch as, by the conditions of the experiment, each rabbit is its own control, it was not thought necessary to repeat the observations on many animals in each experiment. A few typical experiments only need be quoted.

Experiment 1A.—Two rabbits were inoculated with 2 c.c. of a heavy emulsion of SiO_2 in the right flank, and ½ c.c. of turpentine in the left flank. They received the next day 1 c.c. of suspension of tubercle bacilli (human) intravenously.

Rabbit I, killed 6 days after infection, showed early miliary tuberculosis in the lungs, liver and spleen, in which a few bacilli were present. The small silica lesion and the extensive turpentine lesion contained no bacilli.

Rabbit II, killed 26 days after infection, showed numerous lesions in the lungs, liver and spleen. The silica lesion was small and tough. The turpentine one consisted of a broad zone of dry necrosis extending from the groin to the axilla; there was much organizing inflammatory reaction around it.

Microscopically the silica lesion shows a typical tuberculous histology. It consists of a sheet of mononuclear elements mingled with many giant-cells and containing numerous areas of necrosis. Bacilli are present in fair numbers, about as many as in the hepatic lesions, and more than there are in the spleen, though of course nothing like as many as there are in the lung. In the turpentine lesion no bacilli are present in the central necrotic area. In the broad surrounding zone of highly vascular organizing inflammatory tissue, there are a few groups of mononuclear cells with an occasional giant-cell. In these nodes a tubercle bacillus can sometimes be seen, and two or three bacilli are present in a small necrotic focus in one series of sections, but the localization of the infection is trivial in comparison with that of the silica lesion, though the inflammatory reaction is much more widespread and intense.

This experiment was repeated in two rabbits—*Experiment* 3A—using smaller doses of turpentine. The result was the same.

Experiment 2A.—Three rabbits were used, silica and turpentine abscesses being produced in the right and left flanks. Five days later the turpentine and silica injections were repeated, and at the same time the animals received a fairly heavy dose of a bovine strain ("Cockhorn") intravenously. One animal was killed on the 14th day after infection, one on the 17th; the third one died 24 days after infection. The strain of bacilli used in the experiment is highly virulent for rabbits, and produced extensive lesions in all the organs and heavy infection in both silica and turpentine abscesses. The infection in the silica lesion appeared considerably greater than that in the turpentine lesion in Rabbit III, but no definite conclusion can be drawn from the results, and the experiment illustrates very well the fact that accessory factors are of little moment in infectious processes, when the balance between the host and the organism is heavily weighted in one direction or the other.

Experiment 4A.—Three rabbits each received into the right flank 1 c.c. of a saline suspension of SiO_2; into the right axilla 1 c.c. of a 1% solution

of calcium chloride; into the left flank 1 c.c. of a saturated solution of cholesterin in olive oil; and into the left axilla ½ c.c. of a saturated solution of cantharidin in olive oil. ½ c.c. of an emulsion of tubercle bacilli (human) was given intravenously to each animal the following day.

Rabbit II, killed 11 days after infection, showed no bacilli in any of the inflammatory foci, which were well developed. There was a very early infection of the lungs and liver, but bacilli were extremely scanty.

Rabbit I, killed 28 days after infection, showed early lesions with a few bacilli in the organs. The calcium, cholesterin and cantharidin lesions were well developed, but contained no bacilli and showed no tuberculous histology.

The silica focus presented a mononuclear cellular reaction with areas of necrosis in which were a few bacilli.

Rabbit III, killed 37 days after infection. In the *lung* are numerous small cellular foci, the majority of which contain no bacilli. Two large foci contain numerous bacilli.

The *liver* presents a few retrogressing lesions with an occasional bacillus.

The kidney contained numerous cellular foci with very few bacilli. One large one is mostly necrotic and contains about a dozen bacilli per section.

The spleen shows no active lesions.

The silica lesion shows a typical tuberculous histology, with a fair number of bacilli.

The calcium lesion is organizing and is highly vascular, and involves the muscle of the chest-wall. It shows no tuberculous histology and contains no bacilli. The cholesterin lesion is also negative, and the cantharidin lesion could not be found.

The strain of bacilli used in this experiment produces retrogressive tuberculosis in the rabbit. In the organs most of the lesions tend to heal; in only a few chronic foci is the infection spreading. In the silica lesions of rabbits I and III there is undoubtedly progressive tuberculosis, though in the other inflammatory regions there is no infection.

CONCLUSIONS.

If localized inflammatory lesions are produced in the subcutaneous tissues of mice and rabbits, and the animals are subsequently injected intravenously with tubercle bacilli, the increased vascularity of the inflammatory areas renders these more liable to infection than the normal connective tissues. There is evidence that such an infection does take place under these circumstances, when the provocative agent is calcium chloride or turpentine, though only to a very moderate degree, even when the inflammatory reaction is widespread and intense. And there is nothing to indicate that in these "fixation abscesses" the conditions are more favourable to the growth of tubercle bacilli than in uninjured tissues. If, however, the "fixation abscess" is produced by silica, the tubercle bacilli proliferate in it to a most remarkable extent, and in susceptible animals produce a characteristic tuberculous focus. Inasmuch as the intensity and extent of the inflammatory reaction produced by silica is usually much less than when calcium chloride or turpentine are used, vascularity and tissue necrosis can scarcely be the determining factors, and the difference appears to be due to the actual presence of the silica.

Exactly how the silica acts is still uncertain. Experiments which are now in progress suggest that the presence of silica in the protoplasm of a phagocytic cell renders it less able to cope with such tubercle bacilli as it may ingest than normal cells, although it suffers no recognizable damage from the presence of the silica. It is possible, of course, that the vitality of the cell is injured by the silica, although ordinary histological examination shows no evidence of this, but it is also possible that silica itself is a direct stimulant to the growth of the organism. It is hoped that further experiments may throw some light on this question.

SUMMARY.

Tubercle bacilli injected into the circulation of rabbits and mice tend to settle in localized subcutaneous lesions produced by various agents which bring increased vascularity and tissue necrosis. When silica is the provocative agent, the determination of the organisms to the inflammatory focus is invariable, and the bacilli proliferate extensively in such lesions. Inasmuch as similar results are not obtained with other provocative agents, silica appears to have some specific action on the growth of the tubercle bacillus in the tissues.

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EXPLANATION OF PLATE.

Experiment 7A.—Mice injected with 5 mgm. silica into the right flank and 5 mgm. $CaCl_2$ into the left flank. Human T.B. injected intravenously next day. Animals killed 16 days later.

Fig. 1.—Calcium lesion of Mouse 7A/12. There are two or three similar foci in the lesions, but this field was specially chosen as containing more bacilli than any other.

Fig. 2.—Silica lesion of Mouse 7A/12. Healing peripheral lesion. The field was not specially selected. There are many others equally rich in bacilli. In addition, the main silica lesion contains innumerable bacilli.

Fig. 3.—Silica lesion of Mouse 7A/8. Margin of the necrotic focus showing proliferation of tubercle bacilli.

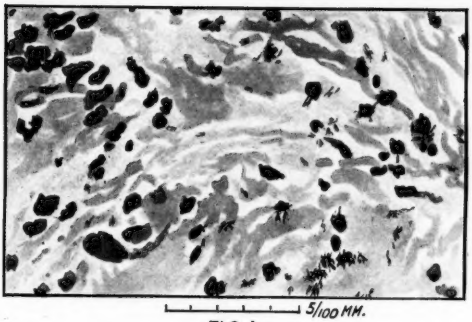

FIG. 1

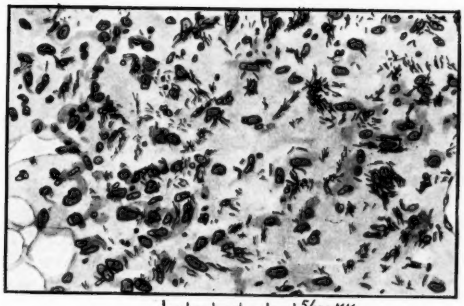

FIG. 2.

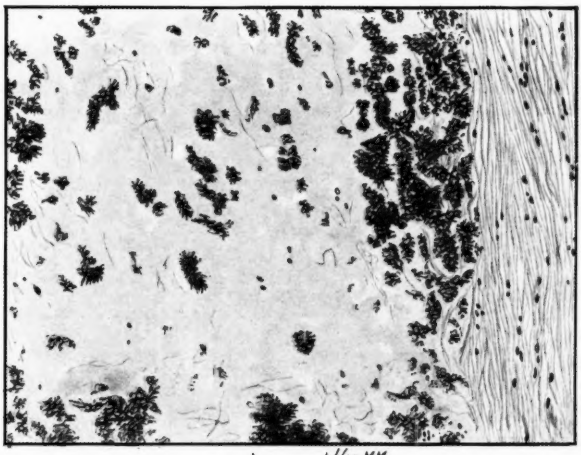

FIG. 3.

Kettle.

UNIV. OF MICH.

THE EFFECT OF THE ADMINISTRATION OF VACCINES ON THE LYSOZYME CONTENT OF TISSUES AND SECRETIONS.

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THE widespread occurrence of lysozyme in the tissues and secretions of the human and animal body has already been shown in papers by Fleming (1922), and Fleming and Allison (1922[1], 1922[2]). Tissues from every part of the human body were tested and found to contain lysozyme in varying quantities; only the cerebrospinal fluid and normal urine and fæces were found to be devoid of bacteriolytic action. The animal tissues examined included those of the dog, rabbit, guinea-pig, pike, frog, earthworm and sea anemone, and all were found to possess bacteriolytic power in some degree, as shown by their action on the indicator organism *M. lysodeikticus*.

The response of the body and particularly of the blood constituents to microbial infection, and after inoculation with vaccines, has been extensively investigated; the increased bactericidal power of the serum and the various immune reactions appearing in it, during the course of infections or of vaccine treatment, have received special attention, but comparatively little work has been done on the investigation of the effect produced on the other tissues and secretions of the body. It was therefore thought to be of interest to inquire whether lysozyme, a bacteriolytic substance already present in the tissues and secretions, could be increased by active immunization with vaccines.

Three series of experiments were carried out on man and animals, using vaccines of (*a*) organisms very sensitive to the bacteriolytic action of lysozyme —namely, *M. lysodeikticus;* (*b*) organisms moderately sensitive to lysozyme, for example, a fæcal streptococcus; and (*c*) organisms almost insensitive to lysozyme action, such as *B. typhosus*, *B. paratyphosus A* and *B. paratyphosus B*.

In the first experiment six normal healthy rabbits were used; the bacteriolytic titre of the tears and blood sera of all six rabbits against *M. lysodeikticus* was tested, commencing with tears diluted 1 in 100 in normal saline, and serum diluted 1 in 10. The tears and sera were diluted 2-fold in series of six tubes each, and to each tube one drop of a thick saline suspension from an agar slope culture of *M. lysodeikticus* was added. The tubes were incubated at 45° C., the optimum temperature for lysozyme action *in vitro*, and readings were taken after three hours. The tears in all cases showed some lytic power (Table I), although the titre varied slightly in the different rabbits; the sera from three of the rabbits showed very weak lytic action, while the sera from the other three were apparently devoid of lytic power in

the dilutions tested. In a series of about 20 rabbits examined, 40 per cent. showed no lytic action on *M. lysodeikticus* in the serum diluted 1 in 10; it was also noted that a low lysozyme content of the tears did not connote a low lytic titre or absence of lysozyme in the serum of the same rabbit, nor was the reverse true.

TABLE I.—*To Show the Variation in the Bacteriolytic Titre of the Tears and Blood-serum of Normal Rabbits before Inoculation. Tested against* M. lysodeikticus.

Rabbit.	Tears diluted 1 in—				Control.	Serum diluted 1 in—				Control.
	100.	200.	400.	800.		10.	20.	40.	80.	
A	±	Tr	—	—	—	—	—	—	—	—
B	AC	±	Tr	—	—	—	—	—	—	—
C	±	Tr	—	—	—	AC	±	Tr	—	—
D	C	C	AC	AC	—	—	—	—	—	—
E	C	AC	±	Tr	—	AC	AC	±	Tr	—
F	AC	±	Tr	—	—	±	±	Tr	—	—

Incubation period—3 hours at 45° C. C = complete lysis. AC = almost complete lysis. ± = lysis well marked. Tr = trace of lysis.

The tears and sera of the six rabbits were tested in a similar manner on four occasions at intervals of four days in order to determine whether there was normally any variation in the bacteriolytic titre of the tears or sera of the individual rabbits; in all cases it was found to vary slightly within narrow limits. It was decided that any well-marked or sustained change in the lytic titre of the tears or sera of the rabbits tested against *M. lysodeikticus* during the course of the experiment should be taken to indicate an alteration in their lysozyme-content. The vaccine used was a living suspension in saline of a 24-hour-agar plate culture of *M. lysodeikticus*, and it was administered intravenously to three of the rabbits (A, C, F), commencing with a dose of 400 million cocci. Inoculation was repeated every fourth day as shown on Chart I, gradually increasing the dose up to 10,000 million cocci. The other three rabbits (B, D, E) were kept as controls, and the tears and blood-sera from all six were titrated every fourth day for lytic action on a saline suspension of *M. lysodeikticus* of constant concentration. Agglutinins appeared in the sera of the three immunized rabbits up to a dilution of 1 in 40 at the end of twelve days, but at no time during the course of the experiment did any agglutinins appear in the tears. Chart I represents the averaged lytic titres of the tears and sera of the three immunized and the three control rabbits tested during the course of the experiment; it will be seen that there was no increase in the lytic power of either the tears or the sera, except for a slight rise in the titre of both tears and sera on the twenty-eighth day; as this rise only occurred on one occasion, and as the tears and sera of the control rabbits also showed a similar rise, the result was probably due to the test suspension of *M. lysodeikticus* being less concentrated than the standard suspension.

Four days after the injection of 10,000 million cocci the three rabbits were given massive doses of *M. lysodeikticus*, 25,000 million cocci being given to each rabbit on each of eight successive days; the rabbits did not appear to

suffer any ill-effects after these doses. The agglutinins in the sera increased up to a titre of 1 in 80, but the tears and sera, tested at intervals up to a period of twelve weeks from the first massive injection, showed no change in lytic power. At this stage one of the immunized rabbits (C) was given 100,000 million cocci intravenously, and ten days later this rabbit and one of the control rabbits were anæsthetized, bled and killed. The spleen, liver, heart, tongue, lungs and kidneys were removed from each, washed rapidly in running water, minced and dehydrated in acetone for 24 hours, after which they were dried in the incubator at 37° C. One gramme of each tissue was ground up in a mortar in 10 c.c. of normal saline and allowed to extract at room temperature

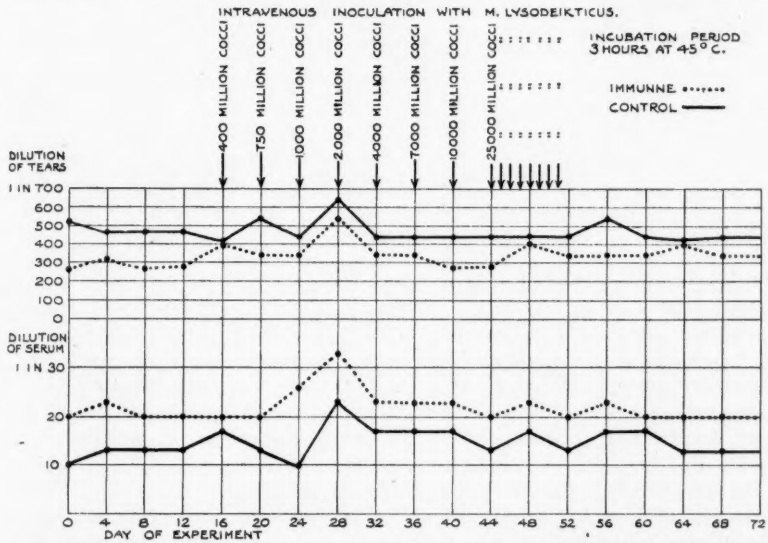

CHART I.—The highest dilution of tears and blood-serum showing bacteriolysis in immunized and control rabbits, tested against *M. lysodeikticus*. (Average of three animals in each case.)

for 48 hours; the extracts were then centrifuged, diluted 2-fold in series, commencing with a dilution of 1 in 25. One drop of a thick saline suspension of *M. lysodeikticus* was added to each tube, including controls, and the series were all incubated at 45° C. for one hour, when readings were taken.

The results obtained are shown in Table II, which gives the highest dilution of the various tissue extracts showing definite signs of lysis, and comparing the lytic power of the tissues of the two rabbits, it is evident that there has been no increase in the lysozyme-content of the tissues of the immunized rabbit. The sera from the blood collected when the rabbits were killed were also tested at the same time, and there was still no change in their lytic power. During the course of the experiment a slight increase was observed in the bactericidal power of the sera of the immunized rabbits, but this increase disappeared on heating the sera to 55° C. for 30 minutes, and was probably due to the production of specific bactericidins.

The results of this experiment show that the lysozyme-content of the body-tissues and secretions is not increased by active immunization with an organism such as *M. lysodeikticus*; as, however, there was very little development of any demonstrable immune bodies following the injection, even of massive doses, of this organism, it was thought possible that it might not act as an efficient antigen. Accordingly a second series of experiments was conducted, this time on a human subject.

TABLE II.—*To Show the Lytic Action of Rabbits' Tissues, "Immunized" and Control, on* M. lysodeikticus.

Tissue.	Dilution in which lysis was almost complete in 1 hour at 45° C.	
	"Immunized" animal.	Control animal.
Lungs	1 in 1600	1 in 3200
Heart	,, *25	,, *25
Liver	,, 50	,, 50
Spleen	,, 3200	,, 1600
Kidney	,, 1600	,, 1600
Tongue	,, 200	,, 400
Cartilage	,, 3200	,, 6400

The organism selected was a fæcal streptococcus which gave the sugar reactions of *S. fæcalis*, and was moderately sensitive to the lysozyme in human tears, being dissolved by them up to a dilution of 1 in 3000 after incubation for 3 hours at 45° C., and it also stimulated the production of antibodies, *e.g.* agglutinins, on injection into the animal body. At the beginning of the experiment, as before, the lytic action of the tears and blood-serum of the subject was tested at intervals on the streptococcus, to estimate the normal variation in titre; this remained remarkably constant, especially in the case of the tears, while the serum showed only a negligible variation. The test suspensions of the streptococcus used in the test were made by washing off a 24-hour agar plate culture into normal saline, and diluting to an opacity as nearly equal as possible to that of an arbitrary suspension of barium sulphate in water. The lytic action was tested using serial 2-fold dilutions of tears, commencing with 1 in 100 and serum 1 in 10, and adding to each tube, with controls, one drop of the streptococcal suspension; readings were taken after incubation for one hour and three hours at 45° C. At the same time the bactericidal power of the tears and serum on the streptococcus was also tested, using tears 1 in 2 and serum 1 in 2, diluted 2-fold up to 1 in 128 and employing a 1 in 1,000,000 dilution in broth of a 24-hour broth culture of the organism. In these tests the technique used was to dilute the tears and serum 2-fold on sterile paraffin slides with capillary pipettes, graduated to 10 c.mm.; the dilutions were made in saline with a control volume of 10 c.mm. of saline. Volumes of 10 c.mm. of the diluted broth culture of the streptococcus were added to each dilution of tears and serum, and to the controls. After being mixed the dilutions were incubated in sealed pipettes for one hour at 37° C. and then blown out on to agar plates divided into sections. The plates were

* Even in this dilution, the strongest used, lysis was only partial.

incubated at 37° C. for 18 hours and the number of colonies on each section was counted.

After the preliminary series of tests of the bacteriolytic and bactericidal powers, which were found to remain remarkably constant, the subject was given hypodermically 10 million streptococci from the killed vaccine of the *S. fæcalis* against which the tears and serum were being tested. The tears and serum were tested at intervals of five days for bacteriolytic and bactericidal action on the streptococcus, while the dose of the vaccine was gradually increased up to 250 million streptococci as shown in Chart II, which demonstrates the highest dilution of tears and serum showing definite signs of lysis, and bactericidal action on the streptococcus after 3 hours' incubation at 45° C.

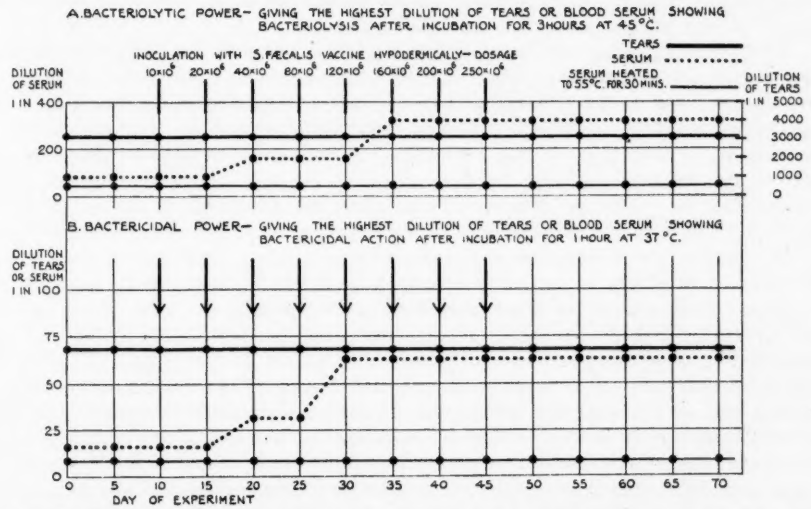
A. BACTERIOLYTIC POWER— GIVING THE HIGHEST DILUTION OF TEARS OR BLOOD SERUM SHOWING BACTERIOLYSIS AFTER INCUBATION FOR 3 HOURS AT 45 °C.
TEARS
SERUM
INOCULATION WITH S. FÆCALIS VACCINE HYPODERMICALLY— DOSAGE
10×10⁶ 20×10⁶ 40×10⁶ 80×10⁶ 120×10⁶ 160×10⁶ 200×10⁶ 250×10⁶
SERUM HEATED TO 55°C. FOR 30 MINS.
DILUTION OF SERUM
1 IN 400
200
0
DILUTION OF TEARS
1 IN 5000
4000
3000
2000
1000
0
B. BACTERICIDAL POWER— GIVING THE HIGHEST DILUTION OF TEARS OR BLOOD SERUM SHOWING BACTERICIDAL ACTION AFTER INCUBATION FOR 1 HOUR AT 37 °C.
DILUTION OF TEARS OR SERUM 1 IN 100
75
50
25
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
DAY OF EXPERIMENT

CHART II.—To show the effect of inoculation with *S. fæcalis* on the lysozyme action of tears and blood-serum in the human subject, tested against *S. fæcalis*.

during the course of the experiment. Agglutinins appeared in the serum at the end of 10 days up to a titre of 1 in 40, increasing later up to 1 in 320, and the bactericidal and bacteriolytic power of the serum also increased; from the beginning and throughout the experiment the action of the serum was tested, both unheated and heated, to 55° C. for 30 minutes to eliminate the bactericidal power. Throughout the experiment there was not observed any increase in the bacteriolytic or bactericidal power of the tears, or of the serum heated to 55° C. for 30 minutes.

The results of this series of experiments therefore lead to the same conclusions drawn from the results of the previous experiment, and also indicate that there is no connection between lysozyme and the phenomena of active immunization.

A final series of confirmatory experiments was carried out, using vaccines of organisms pathogenic for man, which had been found to be almost insensi-

tive to lysozyme action. Six rabbits were used, three being inoculated with vaccines of *B. typhosus*, *B. paratyphosus A* and *B. paratyphosus B* respectively, while the other three acted as controls. The tears and blood-sera of the test animals were tested as before for lytic action on the organisms with which they were to be inoculated, while the controls were also tested in a similar manner with all three organisms. There was no evidence of any lytic action in the tears or sera of any of the rabbits either before or during the experiment, in the course of which the test animals were given intravenous inoculations of 50 million, 100 million, 250 million and 500 million organisms at intervals of 5 days. An increase in the bactericidal power of the sera of the test animals occurred, due to the formation of specific antibodies, but this disappeared on heating the sera to 55° C. for 30 minutes; the tears remained devoid of any bactericidal action on the organism.

In this experiment the tears and sera of the inoculated rabbits were tested both before and during the experiment, not only with the homologous organism, but also with *M. lysodeikticus*, and the strain of fæcal streptococcus used in the previous experiment, in order to determine whether, if there was no increase in lytic action on the pathogenic homologous organism, there might not be an increase in lytic action on other organisms non-pathogenic and susceptible to lysozyme action. There was no evidence of increased lytic action by the sera or tears on any of the organisms, and it was therefore concluded that it is not possible to increase the content of lysozyme in the tissues and secretions of the living organism by active immunization with vaccine.

In a recent article on lysozyme, Bail (1923) points out that Weil (1911) and Suzuki (1911) described the action of leucocytic substances, which appeared to be similar in their properties and action to lysozyme. The latter workers experimented with guinea-pigs, and described the action of leucocytes *in vivo* and *in vitro* on ten strains of organisms derived from the laboratory air and from spring-water. They also attempted to modify the lytic action of the leucocytic substances in the guinea-pigs by inoculation of living vaccines of these organisms intraperitoneally, and subsequent examination of the leucocytic action on the organisms, but they did not succeed in increasing or altering the lytic power of the leucocytic substances. They described the substances as being entirely of leucocytic origin, and did not connect this leucocytic action with any similar action of the other tissues or of the secretions. If the substances described by Weil and Suzuki be accepted as similar to lysozyme, the results obtained from their experiments are confirmed by the results of the experiments described above.

CONCLUSIONS.

The lysozyme content of the tissues or tissue fluids of man or animals cannot be increased by active immunization.

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STUDIES ON THE GROUP SPECIFICITY OF SOME ANTIGENS DERIVED FROM ACID-FAST BACILLI.

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The experiments to be described in this paper have been carried out with the object of trying to determine the limit of the group specificity of some proteid antigens derived from acid-fast bacilli.

We have not taken into account any immunity related to the fatty and waxy coats of the bacilli, and have considered only the proteid portion.

In the experiments at present under discussion we have confined ourselves to the use of the following antigens:

1P. Dried *B. phlei.*
2P. ,, treated with 25 per cent. antiformin.
3P. ,, ,, by Dreyer's (1923) method (diaplyte).
4P. ,, ,, with 50 per cent. antiformin.
5P. ,, ,, with pure antiformin.
6P. ,, subjected to dry distillation.
1T. Dried human tubercle bacilli.
3T. ,, ,, treated by Dreyer's method.

In addition a few antigens of an origin other than that of acid-fast bacilli have been used to test the extent of the group specificity. So far as our experiments went they confirmed the results obtained by other workers.

The mother suspensions of antigens 1P. and 1T. were prepared by making up a 1 per cent. suspension of the dried bacilli in normal saline, the bacilli being ground down very carefully in a mortar.

Antigen 2P. was prepared by heating 5 gm. of the dried bacilli, suspended in 50 c.c. of 25 per cent. antiformin, in a water-bath at 65°C. for 30 minutes, filtering, thoroughly washing with distilled water to remove the last trace of alkali, drying over a water-bath, and making up the dried remainder to a 1 per cent. suspension in normal saline. Antigen 4P. was prepared in a similar manner to the above, with the exception that the mixture was made with 50 per cent. antiformin and the whole heated at 100°C. for 5 minutes. In the case of antigen 5P. pure antiformin was used and the suspension boiled for 8 hours.

For the preparation of antigen 6P. the dried *B. phlei* were subjected to dry distillation, the volatile products being collected in water.

Antigen 3P. and 3T. were prepared according to the method advocated by Dreyer (1923), a 1 per cent. emulsion in saline of the residue being made.

Rabbit high-titre sera were prepared by the injection of some of these antigens. One c.c. of the mother suspension was diluted with 4 c.c. of normal saline, and injected intravenously into a rabbit every 4 or 5 days, the serum being titrated 3 to 4 days after the last injection.

As regards the reaction of the rabbits to the injection of the different antigens, in their production of complement-fixing anticorps, we found very little difference—in fact no more than could be accounted for by the variations to be expected among individual animals. Perhaps the whole bacilli were a little superior to the treated bacilli. From the point of view of the production of a serum of as high a titer as possible or from the point of view of specific therapy, it must be borne in mind that the treated antigens are probably very much less toxic than the whole antigens, and they can be used in massive doses.

The results obtained by complement-deviation tests with the high titre sera will first be considered. The first observation of interest that was made was that the anti-complementary titre of the antigens showed a wide range of difference, the whole bacilli of both varieties absorbing 30 to 40 times the amount of complement absorbed by the antigens prepared with antiformin or by Dreyer's method; an exception was Antigen 2P., which was found to be slightly alkaline. This looked as if the latter kind of antigens would be more favourable reagents for the carrying out of diagnostic tests with diseased animals' sera. However, by titrating the antigens with a more or less minimal dose of high-titre serum but little difference was found between them—in other words, we were using 30 to 40 times as much of antigens 3P., 4P. and 3T. as was necessary to obtain a nearly maximum reaction. The actual figures we obtained will be found in the accompanying Table I.

TABLE I.—*Complement-deviation Tests.*

Antigen.	Anti-complementary titre 1 in—	Antisera: Complement-fixing titre 1 in—				
		1P.	2P.	3P.	1T.	3T.
1P.	150	725	155	116	580	87
2P.	200	116	155	116	72	trace
3P.	6	1160	725	232	580	145
4P.	7·5	1550	290	232	580	145
5P.	5	—	—	0	0	0
6P.	5	—	—	0	0	0
1T.	120	725	155	116	2320	116
3T.	3·8	1550	725	232	1160	174

When tested with high-titre sera one found lack of type specificity. We found a closer relationship between two different defatted antigens than between a whole and a defatted antigen originating from a single variety of bacillus. Thus 3P. and 3T. reacted more nearly alike than 1P. and 3P. Antigen 2P. reacted more like the whole bacilli than like the diaplyte, while antigen 4P. reacted in a similar manner to the diaplyte. This was anticipated, as it was found that microscopically the bacilli in the 2P. antigen retain their bacillary form, and although completely non-acid-fast, many of the granules may stain by Gram; on the other hand, the 4P. antigen is pulped more thoroughly, the bacillary form of the bacilli being almost lost, as in the diaplyte antigen.

On boiling the bacilli for 8 hours with pure antiformin the small solid

residue remaining seems to have lost completely its antigenic properties. The volatile products obtained by dry distillation of the bacilli also have no appreciable antigenic properties. In the complement-deviation test the homologous antigen may show itself to be slightly superior to related forms, but not, in our experience, to any certain degree. In test-tube experiments there is perhaps a little more specificity among the whole bacilli than there is among the defatted ones.

We found the defatted bacilli to be superior to the whole bacilli for the detection of complement-deviating anticorps in the sera of tubercular patients, defatted *B. phlei* being as efficacious as defatted tubercle bacilli. Syphilitic and some other control sera were invariably negative with all the antigens, as were the sera of a dozen tuberculous guinea-pigs. We were also unable to detect the presence of antigen in the blood of these tuberculous guinea-pigs by the use of high-titre immune sera of the rabbit, although it could be detected in human sera from tuberculous patients.

TABLE II.—*Agglutination Tests.*

Antigens.	Antisera: Agglutinating titre 1 in—				
	1P.	2P.	3P.	1T.	3T.
1P.	25	0	12	40	trace
2P.	200	320	260	160	80
3P.	200	40	100	160	80
4P.	200	40	80	160	80
1T.	50	trace	trace	50	trace
3T.	200	25	100	160	80

Readings taken after incubation at 37° C. for 24 hours.

The results obtained with the agglutination reaction, which will now be discussed, were very similar to those obtained with the complement-deviation reaction (Table II). There was practically no specificity except within the acid-fast group. Whole bacilli, whether tubercle or *phlei*, gave much the same titres with each antiserum used, and the titre given by the whole bacilli was considerably less than that given by the treated bacilli. Defatted tubercle bacilli and *B. phlei* were equally efficacious as antigens no matter what antiserum was used, and the antigens prepared with antiformin or with formalin-acetone were equally sensitive. The formalin-acetone antigens are, in our opinion, slightly superior to those prepared with antiformin, as spontaneous sedimentation is on the whole a little longer in taking place.

The most specific agglutination reaction we have obtained is the reaction of a 2P. antigen with its homologous high-titre serum. We are unable to offer a feasible explanation as to why there is more specificity here than with a 1P. or 3P. antiserum with their homologous antigen; we have noted this fact on several occasions, and it will be interesting to see to what degree a 2P. antiserum reacts with a 2T. antigen, and *vice versâ*.

We have treated two dozen tubercular guinea-pigs therapeutically with a *B. phlei* antigen prepared according to the technique of Dreyer, but have been unable to convince ourselves that any appreciable beneficial effect was

obtained. As regards the production of local necrosis, all defatted antigens which we have used have produced local caseous formation at the site of injection on occasions, although the tendency to do so is very much less than is the case with the whole bacilli. We have seen, however, that our antigens 2P. and 4P. contain free lipoidal substances; on the other hand, the diaplyte antigens are presumably fat-free, so that it remains a doubtful question as to whether the contention that these substances are the essential factor in the production of necrosis is correct or otherwise.

CONCLUSIONS.

1. The inoculation into rabbits of whole, dried acid-fast bacilli or defatted bacilli leads to the production of sera with an approximately similar titre, *i. e.* as regards complement-deviation and agglutination anticorps.

2. When dealing with high-titre sera, defatted antigens are slightly superior to the whole bacilli as antigens in complement-deviation tests; they are very much superior as agglutinable substances.

3. For the diagnosis of human tuberculosis by the complement-deviation test, it appears from the small number of reactions we have carried out that the defatted antigens of *B. phlei* or *B. tuberculosis* are markedly superior to the whole bacilli; the same applies to animal's sera the titres of which are very low.

4. The anti-complementary titre of whole bacilli is 20 to 40 times that of the defatted bacilli, but in carrying out the complement-deviation test it seems that the extra amount of defatted antigens which can with safety be used is really only of advantage when the quantity of anticorp available is small. The end-point when high-titre sera are used is not much influenced by the amount of antigen employed, within wide limits.

5. There is no type specificity to any appreciable degree, an anti-tubercle bacillus serum reacting as well with a *B. phlei* antigen as with a tubercle bacillus antigen in both complement-deviation and agglutination tests.

6. There appears to be more difference in antigenic properties between any defatted antigens and any whole bacillary antigen, than between any two defatted antigens or any two whole bacillary antigens.

7. Group specificity is strong, it being almost impossible to treat acid-fast bacilli in a manner severe enough to destroy the group antigens without completely destroying the organic complex.

8. It seems probable that the group antigens reside in the proteid portion of the bacilli rather than in their lipoidal covering.

9. In tuberculous guinea-pigs no appreciable beneficial effect has been noted by the use of defatted *B. phlei* vaccines. All the antigens we have employed have produced in animals local caseation under certain circumstances, although to a much less degree, in the case of a defatted bacilli as compared with whole bacilli.

We regret that our experiments are so incomplete, but owing to unforeseen circumstances it has been found impossible to continue the investigations for the present.

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AN INVESTIGATION INTO THE ÆTIOLOGY OF DENTAL CARIES: II. THE BIOLOGICAL CHARACTERISTICS AND DISTRIBUTION OF *B. ACIDOPHILUS ODONTOLYTICUS*. III. FURTHER EXPERIMENTS ON THE PRODUCTION OF ARTIFICIAL CARIES.

JAMES McINTOSH, W. WARWICK JAMES AND P. LAZARUS-BARLOW.

WITH A NOTE ON THE PRODUCTION OF MALIC ACID BY *B. ACIDOPHILUS ODONTOLYTICUS*. By E. C. DODDS.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication April 27th, 1924.

IN a former paper we (1922) showed that a micro-organism belonging to the *acidophilus* group, to which we gave the name *B. acidophilus odontolyticus*, played a very important part in the ætiology of dental caries. With pure cultures of this microbe it was possible to produce an "artificial" caries which was indistinguishable from the "natural" condition. In the present article further details are given of *B. acidophilus odontolyticus*, its relationships and distribution in nature, while the production of experimental caries is further discussed.

BIOLOGICAL CHARACTERISTICS AND DISTRIBUTION OF *B. ACIDOPHILUS ODONTOLYTICUS*.

B. acidophilus odontolyticus, as we have previously shown, belongs to the *acidophilus* group described by Moro. This group consists of a number of individual types, as does the colon-typhoid group, the common feature of the former being a high resistance to acid. The two types which we described are characterized by a high acid resistance and a high acid production. Type I is a long thin bacillus $0{\cdot}75\,\mu \times 2\text{–}3\,\mu$ which occurs singly or in chains. Type II is a shorter bacillus usually occurring in chains.

Further observations on the morphology of these types have confirmed this, and in the case of Type II some additional details are added.

The organism is a bacillus measuring approximately $0{\cdot}5 \times 1\text{–}2\,\mu$ which usually occurs in short chains. It is non-motile, Gram-positive, an aërobe and facultative anaërobe; in most cases there is uniform turbidity in broth, but occasionally the growth settles to the bottom of the tube. Colonies on agar often take 48 hours to appear; they are 0·5–1·0 mm. in diameter, round, greyish and opaque, with a finely granular appearance under the low power. The organism often shows considerable pleomorphism, coccal forms appearing mixed with the bacillary in the same chain in the more alkaline media, whereas almost always the bacillary forms alone appear in the acid media. Before the fact of this pleomorphism was realized several cultures had been

discarded owing to the failure to separate in pure culture, after repeated attempts, what was thought to be a bacillus and a streptococcus; in these, the cultures on agar plates appeared pure under the low power, but stained films did not apparently bear this out. In 61 per cent. of the cases in which the organism was isolated it was found in some cultures to resemble a streptococcus so closely in morphology, that it was only after repeated subculture that the organism was finally classified as *B. acidophilus odontolyticus II.* In these cases it was described at various times as "streptococcus-like," "coccobacillary," "? streptococci" or "diplococcal forms," and at times a chain of what appeared to be a streptococcus would have one or more definite bacilli in the middle. This 61 per cent. represents 11 cases; in 7 of these the coccal forms did not appear in broth below pH 4·5, in one case not below pH 4, but in 3 cases they appeared in broth of pH 2. It would seem therefore that the organism is definitely a bacillus, but able to produce coccal and diplococcal forms in the more alkaline media used during isolation.

The Fermentation and Agglutination Reactions of B. acidophilus odontolyticus *Isolated from Different Sources.*

In our first article we noted that *B. acidophilus odontolyticus* was to be found in most mouths, so that the infective agent of caries is practically always present in the mouth. Further observations were made in this direction with the idea of ascertaining the normal habitat of these bacilli, and the results are detailed below.

The attempts to isolate *B. acidophilus* from the different sources were made by inoculation into broth of a reaction of pH 3·5. All cultures from water and soil gave negative results; in the case of 8 persons whose teeth varied in their condition from open cavities to those with no active caries at the time of experiment, *B. acidophilus odontolyticus I* was isolated in pure culture in every case; each strain, however, fermented saccharose, which was not the case with the great majority of strains isolated from carious dentine. In order to determine whether this might be due to habitat, 19 strains which had been isolated from three to twelve months previously and kept subcultured on broth agar were inoculated into saccharose. When first isolated none of these strains fermented saccharose even after prolonged incubation, but now 11 formed acid and no gas, 2 formed a small amount of acid, and 6 formed no acid. It thus appears that there is an even greater variation in the behaviour of the organism towards saccharose than was found in the first place, since a large proportion of strains tested ferment the sugar after prolonged subculture. Fifty strains of the group were isolated from the following sources:

Human fæces—adult	18 cases
„ „ children	10 „
Stomach contents	12 „
Milk	5 „
Rats' fæces	4 „
Monkeys' fæces	3 „
Lacteol and saurin tablets	2 „

and gave the following sugar reactions:

	Glucose.	Lactose.	Saccharose.	Maltose.	Mannite.	Dulcite.	Dextrin.	Milk.	Raffinose.	Salicin.	Inulin.	Remarks.
Human fæces (adult).	A	A	A	A	A	O	O	AC	A	A	O	Large regular colonies.
	A	A	A	A	O	O	O	AC	O	A	A	—
	A	A	A	A	A	O	O	AC	O	A	A	Crinkled edges.
	A	A	O	A	O	O	O	AC	O	A	O	Smooth colonies.
	A	O	O	A	O	O	A	O	...	...	...	—
	A	A	A	A	O	O	O	O	A	O	A	Smooth very small colonies.
	A	A	A	A	O	O	A	AC	A	O	O	Crinkled edges.
	A	A	A	A	O	O	A	A	O	O	O	„ „
	A	A	A	A	O	O	A	AC	A	A	O	„ „
	A	A	A	A	O	O	A	AC	O	A	O	Smooth edges.
	A	A	A	A	O	O	A	AC	A	O	O	„ „
	A	O	O	A	O	O	...	O	A	O	O	Resembles *Staphylococcus aureus*.
	A	O	A	A	A	O	...	O	O	O	A	Smooth edges.
	A	A	O	A	O	A	A	AC	O	O	A	„ „
	A	O	O	A	A	O	A	AC	O	O	O	Speckled colonies.
	A	A	A	A	A	O	A	AC	A	A	A	„ „
	A	A	A	O	O	O	A	AC	O	O	O	„ „
	A	A	O	A	A	O	A	AC	A	O	A	Small, smooth colonies.
Human fæces (children).	A	A	O	A	O	O	O	A	A	A	O	Smooth edges, large.
	A	A	A	A	O	O	O	O	...	...	...	—
	A	A	A	A	O	O	O	AC	O	O	A	Smooth edges, small.
	A	A	A	A	O	O	A	AC	A	A	A	Crinkled edges.
	A	A	A	A	O	O	O	AC	A	O	O	Smooth edges, large.
	A	A	A	A	O	O	A	AC	A	O	A	„ „ „
	A	A	A	A	O	O	O	AC	O	O	O	Crinkled edges.
	A	A	A	A	O	O	O	AC	A	O	O	Smooth edges.
	A	A	O	A	A	O	O	AC	O	O	O	—
	A	A	A	A	A	O	O	AC	A	O	O	Smooth edges, small.
Stomach contents.	A	A	O	A	O	A	A	AC	O	O	O	Resembles streptothrix.
	A	A	A	A	O	O	...	AC	A	...	...	Resembles *B. acidophilus odontolyticus* I.
	A	A	A	A	A	...	...	AC	O	...	...	Short bacilli.
	A	A	A	A	O	O	...	AC	A	...	...	Resembles *B. acidophilus odontolyticus* I.
	A	A	O	A	O	O	...	A	A	...	...	Ditto.
	A	A	O	A	O	...	...	AC	A	...	...	Short bacilli resembling *B. acidophilus odontolyticus* I.
	A	A	A	A	O	...	...	AC	A	...	...	Ditto.
	A	A	A	A	O	...	...	AC	A	...	...	Long bacilli in long chains.
	A	A	O	A	O	...	...	AC	A	...	...	„
	A	O	A	A	A	...	...	AC	O	...	...	Short thick bacilli. "
	A	A	A	A	O	...	...	AC	A	...	...	„ „ „
	A	A	O	A	O	...	...	AC	A	...	...	Short bacilli resembling *B. acidophilus odontolyticus* I.
Milk.	A	A	A	A	A	O	O	AC	A	O	O	Large regular colonies.
	A	A	A	A	O	O	A	AC	O	O	O	—
	A	A	O	A	A	O	O	AC	O	A	O	Smooth edges.
	A	A	A	A	O	O	O	AC	A	O	...	Crinkled edges, large.
	A	A	A	A	O	...	...	AC	O	...	...	Short bacilli.
Fæces (rats).	A	O	A	A	O	O	A	O	O	O	O	Smooth, very small colonies.
	A	A	A	A	O	O	A	AC	...	...	...	—
	A	A	A	A	A	O	O	AC	A	O	...	Crinkled edges.
	A	A	A	A	A	O	O	AC	O	O	A	—
Fæces (monkey).	A	O	A	A	O	O	A	O	O	O	A	Crinkled edges large.
	A	O	A	A	O	O	A	O	O	O	O	„ „ „
	A	A	A	A	A	O	A	A	O	O	O	Speckled colonies.
Lacteol and saurin.	A	O	A	A	A	O	...	O	A	A	A	Resembles *Staphylococcus aureus*.
	O	A	A	A	O	O	A	AC	O	O	A	No growth on agar.

A = Acid production. AC = Acid and clot. O = No change.

Summarizing these reactions as percentages, the following figures are obtained:

Glucose.	Lactose.	Saccharose.	Maltose.	Mannite.	Dulcite.	Dextrin.	Milk.	Raffinose.	Salicin.	Inulin.	
100	77·7	66·6	94·3	33·3	5·5	66·6	77·7	44·5	39	39	adults.
100	100	80	100	20	0	20	80	60	20	30	children.
100	91·7	58·3	100	16·6	25	...	100	75	...	...	stomach.
100	100	80	100	40	0	25	100	40	25	0	milk.
100	75	100	100	50	0	50	75	33	0	50	rats.
100	33·3	100	100	33·3	0	100	0	0	0	33·3	monkeys.
50	50	100	100	50	0	...	50	50	50	100	tablets.
98·1	81·5	74	98·1	33·3	4·4	56·4	75·9	52·9	28·2	37·8	mean of all cases.

In the case of *B. acidophilus odontolyticus*, saccharose was only fermented in 4 cases out of 38 by Type I when first isolated and by 14 out of 18 cases by Type II; no strains fermented dulcite or dextrin, and it was the exception for any strain to ferment raffinose. Unfortunately the strains of *B. odontolyticus* were not tested as to their action on salicin and inulin. From the above figures certain resemblances and differences are seen between *B. acidophilus odontolyticus* and other members of the group; Type I of the former ferments saccharose less readily, whereas Type II ferments it in approximately the same percentage of cases; on the whole no strains ferment dulcite, and while *B. odontolyticus* has no action on dextrin and practically none on raffinose, these two sugars are fermented by over half the strains isolated.

Type I Serum.

	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Homologous organism	3	3	2	2	2	2	2	2
Milk	3	2	2	2	1	1	1	1
,,	3	3	2	2	2	1	0	0
,,	3	2	2	2	2	1	1	1
,,	2	1	1	1	1	0	0	0
,,	2	2	1	0	0	0	0	0
Stomach contents	3	3	2	1	1	1	1	0
,, ,,	3	2	2	1	1	0	0	0
,, ,,	3	2	2	2	1	1	1	1
,, ,,	2	1	1	0	0	0	0	0
,, ,,	2	1	0	0	0	0	0	0
,, ,,	2	1	1	0	0	0	0	0
,, ,,	3	2	2	1	1	0	0	0
,, ,,	3	2	1	1	1	0	0	0
,, ,,	3	3	2	2	2	1	0	0
Fæces, adult	3	3	2	1	0	0	0	0
,, child	3	3	2	2	1	0	0	0
,, monkey*	2	1	0	0	0	0	0	0
,, rat	0	0	0	0	0	0	0	0
,, ,,	0	0	0	0	0	0	0	0

* Monkey being given *B. acidophilus odontolyticus* by the mouth in attempt to cause "artificial caries" *in vivo* as described on p. 182.

Type II Serum.

Homologous organism	3	3	3	3	3	2	2	2
Milk	3	2	1	1	0	0	0	0
„	3	2	2	1	1	1	0	0
„	2	1	1	1	1	0	0	0
Stomach contents	3	3	2	2	1	0	0	0
„ „	2	1	1	0	0	0	0	0
„ „	2	2	1	1	0	0	0	0
„ „	3	2	2	2	2	1	1	1
„ „	3	2	1	1	0	0	0	0
Fæces, adult	3	3	2	1	1	0	0	0
„ child	3	3	3	3	3	2	1	0
„ monkey*	3	2	1	0	0	0	0	0
„ rat	0	0	0	0	0	0	0	0
„ „	0	0	0	0	0	0	0	0

3 = complete agglutination; 2 = moderate; 1 = trace; 0 = no agglutination.

The general unreliability of the fermentation reactions in the case of *B. acidophilus* suggested the application of the agglutination test to differentiate the various types. Nineteen of the above strains were put up with the antisera of Types I and II, and also tests for absorption of agglutinin carried out (see tables, Type I Serum and Type II Serum).

In testing for absorption of agglutinins, equal parts of emulsion and antiserum were mixed, incubated for half an hour, centrifuged after standing one hour, and the serum put up with the homologous organism. Here again the results were not very satisfactory, as most strains absorbed the agglutinins in the antiserum of both types. *B. bulgaricus*, however, when tested in this way showed no absorption.

From the above it is seen that members of the *acidophilus* group isolated from various sources have a moderately close relationship, those isolated from milk and human sources bearing a closer relation to *B. acidophilus odontolyticus* than do those isolated from the intestinal tracts of monkeys and rats when compared by agglutinating properties; as regards the sugars, raffinose and dextrin and to some extent saccharose appear to be less easily fermented by *B. odontolyticus* than by other members of the group.

Production of Malic Acid by B. acidophilus odontolyticus.

In a series of chemical tests made by Dr. Dodds on the acids produced by *B. acidophilus*, it appears that the principal acid produced is malic acid. In the samples examined, at times as high a percentage as 0·7 was found in the case of malic acid. The next highest acid was lactic, with about 0·01 per cent. Full details of the methods of analyses are given in an appendix supplied by Dr. Dodds.

* Monkey being given *B. acidophilus odontolyticus* by the mouth in attempt to cause "artificial caries" *in vivo* as described on p. 182.

Pathogenicity to Animals and Capsulation.

As regards the pathogenicity to animals, the rabbits used for the production of the antisera received very large numbers of living organisms both intraperitoneally and intravenously without any ill effects. Three mice and 3 guinea-pigs were also inoculated intraperitoneally with the following quantities of a 2000 million per c.c. emulsion made from 24-hour agar slope cultures of 3 strains: mice, 0·3 c.c., 0·5 c.c., 0·6 c.c.; guinea-pigs, 1·0 c.c., 1·5 c.c., 2·0 c.c. In no case was any ill-effect observed.

The organism has not been seen to possess a capsule when studied by Baker's (1920) method or otherwise.

Thermal Death-point, Growth in Alkaline Broth and Action of Disinfectants.

Twenty-five minutes at a temperature of 56°C. sufficed to kill the organism in every case. The degree of alkalinity of the broth in which the organisms ceased to grow was measured approximately according to the table published by McIntosh and Smart (1920); a measured 10 c.c. of broth, pH 7·6, was taken and various quantities of N/1 NaOH added. Growth was stopped when an amount of N/1 NaOH between 0·075 and 0·1 c.c. was added—that is, between pH 9·1 and pH 9·6.

The tests on disinfectants were carried out on the Rideal Walker lines, but as the organism failed to grow in the broth recommended by them, glucose marmite broth was used, and as the organism is a slow-growing one, 4 days' incubation was allowed before reading the results. The original emulsion was 24 hours old. Phenol, thymol, formalin and some of the essential oils were tested as to their power to allow growth to take place after 2½ and 5 minutes, but not after 7½ minutes. To determine whether the type of broth used made any difference to the action of the disinfectant, a known strain of *B. typhosus* was grown in the glucose marmite broth and submitted to the test with the same result as when the usual broth was employed. The results when using phenol were not at all consistent; in every case, however, the organisms were killed in 7½ minutes by 1/50 and 1/60 dilutions, and in only one case with the 1/50 and 2 cases with the 1/60 dilution was there any growth after 2½ and 5 minutes. A dilution of 1/70 nearly always killed the organisms in 2½ minutes. As regards a 1/80 dilution there was growth after 2½ minutes in just over 50 per cent. of cases, with a drop to just over 25 per cent. after 5 minutes, and to 20 per cent. after 7½ and 10 minutes. A 1/90 dilution allowed growth after 2½ minutes in 66 per cent. of cases, dropping to 50 per cent., 33 per cent., and 20 per cent. after 5, 7½ and 10 minutes respectively. A saturated solution of thymol in distilled water was used as a stock solution; this had a strength of 1/1200. In a series of 6 tests carried out growth occurred in the following number of cases:

	2½.	5.	7½.	10 minutes.
1/1200	0	1	0	1
1/1600	0	0	0	0
1/2000	2	1	1	0
1/2400	4	1	3	1
1/2800	4	3	3	3

The essential oils used were oils of cloves, peppermint, rosemary and eucalyptus. A quantity of the oil and distilled water was shaken up in the shaker till a very fine emulsion was obtained; this was kept as a stock solution, and for the purposes of the test a portion was taken and centrifuged and the clear solution used. This was reckoned to be a saturated solution of the oil in water. Saturated, three-quarters saturated and half saturated solutions were used; in all cases oil of cloves had killed the organisms in 2 minutes whereas the other oils gave inconsistent results, but all appeared to have some bactericidal action, approximately the same in all. If the saturated solution were kept for a few days with only a cotton-wool plug, the germicidal action was much decreased, a three-quarters saturated solution of oil of cloves allowing growth to take place after 10 minutes' action. Formalin gave by far the most consistent results; apart from the first test, which was negative throughout, a 1/10 dilution of formalin killed the organisms in every case in 2½ minutes, whilst dilutions from 1/20 to 1/50 did not kill the organisms in 10 minutes.

The dilutions of the above disinfectants necessary to kill *B. acidophilus odontolyticus* are all too strong for use in the mouth with the exception of thymol, which can be used in a saturated solution. This was done in the following manner: the mouth was thoroughly rinsed with a small quantity of normal saline which was then inoculated into broth pH 3·5; a saturated solution of thymol in water was then taken into the mouth and held there for 2½ minutes. The mouth was rapidly rinsed with saline and a second tube of broth inoculated as above immediately afterwards; two other tubes were inoculated ½ hour and 1½ hours later; in some cases the last tube was inoculated after a meal. After 24 hours' incubation 1 loopful from each tube was inoculated into an agar shake culture and a second spread over an agar plate. The colonies on the latter were not actually counted, but in 6 cases the results were proportional to the signs in the table below:

Before.	Immediately after.	½ hour after.	1½ hours after (no meal).	1½ hours after (meal).
++	−	+		++
++	−	−−	+	
++	−	−−	++	
++	−−	−−−*		+++
++	−	+	++	
++	−	+	+	

* − − − = no colonies on agar plate or in shake culture.

In every case there was a very definite drop in the number of colonies immediately after using the thymol, and this in some cases continued up to half an hour. At the end of an hour and a half, however, the number of colonies was, generally speaking, as great as before the thymol was used, and this was so whether a meal were eaten between the inoculation of the third and fourth tubes or not.

FURTHER EXPERIMENTS ON THE PRODUCTION OF ARTIFICIAL CARIES.

In vitro.

In our former paper the results of experiments to produce caries artificially were set forth; these were the production of a condition microscopically

indistinguishable from caries as it occurs in the mouth except for the fact that the lesion started from the pulp-cavity side. Further experiments have been carried out using ordinary broth cultures instead of glucose broth, and also the roots of the teeth or almost the whole tooth has been covered with celluloid varnish so that the organisms would have to decalcify the enamel before reaching the dentine. Of various substances tried celluloid varnish proved the best, as it neither inhibited the growth nor peeled off. Whereas the teeth were very markedly affected in 14 weeks and under when using glucose broth, 33 to 40 weeks in ordinary broth only produced a small degree of artificial caries. In some cases the enamel alone was affected, in others the dentine also had been attacked in the same manner as in previous cases. In those cases in which the whole tooth was covered with celluloid varnish, except for a small area of enamel, the picture of "natural" caries was reproduced in every respect; the organisms destroyed the enamel, decalcified part of the dentine and passed down the dentinal tubules for a short distance (Figs. 1 and 2).

The control experiment with *S. salivarius* in glucose broth showed complete destruction of the enamel on the incising edge and considerable destruction of the dentine, but no organisms were found in the tubules as is the case when *B. acidophilus odontolyticus* is used; nor were any liquefaction foci found. The destruction appeared to be due solely to the action of the acid on the tooth, this organism apparently having no power of entering the dentinal tubules and destroying them. The organism was allowed to act on the tooth for 36 weeks.

In vivo.

In several instances gold bands were fixed round the upper central incisors of rabbits, and a paste of biscuit and glucose broth culture of *B. acidophilus odontolyticus* placed between the teeth and the band twice a week. Rabbits, however, were found to be unsuitable animals for the experiment owing to the rapid rate of growth of the incisors; only in one case was any microscopic lesion found, although in two other cases opacity of the enamel was noticed. In the one case just referred to there was, microscopically, a definite affection of the enamel at one point with the cementum immediately opposite also affected. Next, a monkey was fed twice daily on a mixture of middlings, white flour, oats and glucose marmite culture of the organism. After 3 months there was no evidence of caries; at this time the enamel of 2 opposing molars was drilled through to the dentine, the upper on the buccal surface and the lower on the occluding surface, and a paste of the organisms and biscuit in glucose broth introduced. At the end of another month there was still no evidence of caries in any of the teeth. A gold band was then fixed round the upper incisors and the animal fed as before, except that a small quantity of acid sodium phosphate was added to the mixture in an attempt to render the saliva, which had a reaction of about pH 7, more acid. After the band had been in place about 7 weeks it became detached and was not replaced; on examining the teeth 16 weeks after the band was first applied there was seen to be some discoloration of the central incisors, and the molar drilled on the buccal surface was found to have a larger cavity than that made

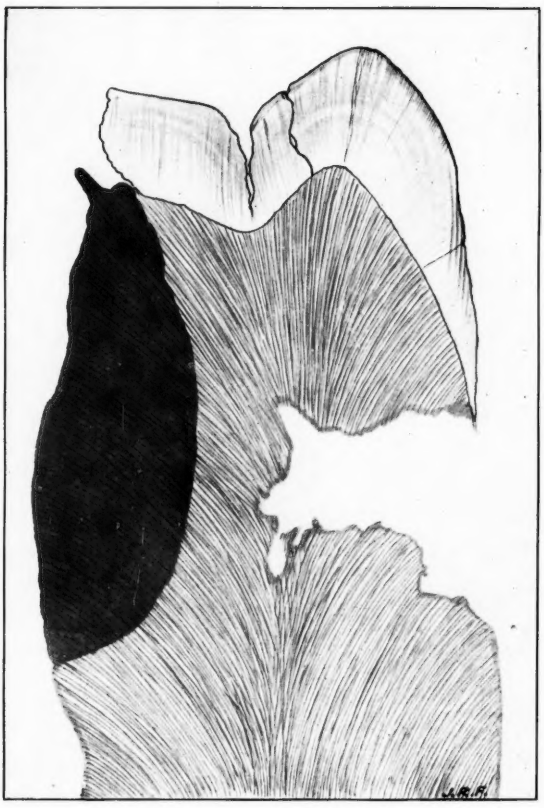

FIG. 1.

Ground section of tooth showing destruction of enamel and decalcification of dentine. Stained by Gram. The dark area shows decalcified dentine, which retains the stain.

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FIG. 2.
Decalcified and bacterially invaded area. × 1000.

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by the drill. The dentine, however, was not softened in any degree, so that the tooth could not be considered definitely carious.

SUMMARY OF RESULTS.

(1) *B. acidophilus odontolyticus* Type II shows considerable pleomorphism, short coccal forms appearing in the more alkaline media.

(2) *B. acidophilus odontolyticus* Type I was isolated in pure culture from all cases of saliva tested.

(3) Organisms belonging to the group were never found in soil, or water, but were isolated from various other sources.

(4) The frequent occurrence of this bacillus in milk suggests this medium as an important source of infection.

(5) Members isolated from human sources were agglutinated to a small degree by the antisera of *B. acidophilus odontolyticus*; those isolated from animals were not agglutinated.

(6) Malic acid was found to be the acid produced in largest quantity by *B. acidophilus odontolyticus.*

(7) *B. acidophilus odontolyticus* is non-pathogenic to animals; it possesses no capsule.

(8) Thirty minutes at 56°C. kills the organism; it fails to grow in alkaline broth between pH 9 to pH 9·5.

(9) The concentration of most disinfectants suitable for use in the mouth and necessary to kill the organism in 5 minutes is too great to be practicable. Thymol 1 in 1200 can be used.

(10) Artificial caries can be produced in ordinary broth by the prolonged action of *B. acidophilus odontolyticus* on a tooth. By suitable methods the picture of " natural " caries can be reproduced in all respects.

(11) Experiments to produce caries artificially in rabbits have failed. There is slight evidence of success in the case of a monkey.

We wish to express our indebtedness to Mr. J. Q. Rowett, who has generously defrayed a large part of the expenses of this research.

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APPENDIX: PRODUCTION OF MALIC ACID BY *B. ACIDOPHILUS ODONTOLYTICUS.*

E. C. DODDS.

As qualitative or quantitative tests on the broth were quite impossible owing to the colour and protein content, it was decided to distil the broth, in the hopes that some of the acid bodies present would pass over.

One litre of broth, which had been cultured with the organism for a fortnight (average pH 2·5), was distilled until the volume was reduced to 40 c.c. The clear distillate (950 c.c.) was found to be strongly acid, whilst the residue was only faintly so. This experiment suggested that the acidity was not due to lactic acid, since this body is non-volatile.

The titrable acidity of the distillate was found to be about N/10. The residue from the distillation was shaken with ether 4 times, and the ethereal extract was evaporated to dryness, leaving a drop or so of syrupy liquid. This was found to be strongly acid in reaction, and to give the tests for lactic acid. To determine the nature of the acid in the distillate, it was neutralized with N/10 sodium hydroxide, and evaporated to small bulk, when crystals separated out. These were collected and dried. An aqueous solution was made, and submitted to a qualitative analysis for organic acids. It was found that the crystals were composed mainly of sodium malate, since the following reactions were positive:

1. No precipitate with ammonium chloride.
2. Precipitate with $CaCl_2$ on warming, or on the addition of alcohol.
3. On the addition of a solution of β-naphthol and sulphuric acid a greenish colour developed, which on heating turned red, and on the addition of water, orange.
4. The ethyl ester was made, and was found to possess the characteristic cyder-like odour.
5. A specimen of the acid was prepared in crystalline form, and was found to melt at 100°C. A mixed melting point determination was made with pure malic acid and there was no alteration in the melting point.

In the following table will be found details of the analyses, with rough calculations from titration figures, of the percentages of malic and lactic acids present:

No.	Vol. of broth.	Vol. of distillate.	Vol. of residue.	Titrable acidity of distillate c.c. N/10 %.	Calculated percentage of malic acid.	Calculated percentage of lactic acid.
1	998 c.c.	952	40	98	0·6	0·01
2	1000 „	969	27	100	0·67	0·008
3	978 „	964	20	102	0·68	0·007
4	995 „	950	40	131	0·7	—
5	500 „	490	7	121	0·69	—
6	500 „	450	32	110	0·68	—

The percentages of acids are based upon the titration figures, and can therefore only indicate very roughly the quantities present. From these analyses it appears that practically all the acidity is due to malic acid. Lactic acid is only present in traces.

STUDIES OF THE IMMUNIZING PROPERTIES OF BACTERIAL ANTIGENS PREPARED AFTER VARIOUS METHODS. I.

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THE present studies were undertaken to throw some light on the comparative immunizing values of various antigens prepared from the so-called endotoxin-producing group of bacteria.

A large number of antigens are at the present time being used for therapeutic purposes, but, with the exception of some work of Perry and Kolmer (1913), the comparative antigenic value of these products has not been actually determined. These authors published a list of bacterial vaccines, prepared after various methods, which showed that agglutination and complement-fixation bodies were produced in highest degree by the administration of living and autolysed vaccines followed in order by mercurophen-killed, trikresol-killed, heat-killed and alcohol-killed sensitized sediment vaccines. The various vaccines used as antigens in the complement-fixation tests were arranged as follows: living and autolysed; mercurophen-killed; trikresol-killed; alcohol-killed sensitized sediment and heat-killed. They obtained the immune sera by injecting rabbits subcutaneously with doses of the antigen equivalent to 1,200,000,000 bacilli per 60 kgm. of weight of rabbit. Agglutination and complement-fixation tests were used to determine antigenic properties of the vaccines. The authors state that "while the production of agglutinin and lysin is not acceptable at the present time as a strict index of actual protection, nevertheless such production may be accepted as an index of the antigenic properties of the various vaccines and the degree of specific response elicited by them."

As a result of previous unpublished experiments on broth cultures carried out by us at the suggestion of Sir Thomas Horder, of London, which brought out points rather opposed to existing current opinions in regard to antigenic properties of the broth filtrates (see Experiment 1), it was decided to conduct further experiments on filtrates prepared in a similar manner, and to augment the work by testing the immunizing properties of other antigens prepared from broth cultures as well as from cultures on solid media.

In most instances antibody response was determined by the agglutination and complement-fixation tests, but with some of the antigens active and

passive protection tests were carried out, and in one experiment the bacteriotropin test was utilized.

TECHNIQUE.

The method of producing immune sera differed materially from that of Perry and Kolmer, as it was previously found by one of us (Ferry and Noble, 1918) that individual rabbits immunized intravenously exhibited less variation than those immunized subcutaneously. Rabbits of 6 pounds weight were given three intravenous injections of the antigen preceded by one desensitizing injection given subcutaneously, and bled to death five days after the last injection. The first or desensitizing subcutaneous dose was 0·5 c.c., followed by one dose of 1 c.c. and two doses of 2 c.c. each, given intravenously three days apart. Two and, in some instances, three rabbits were injected with each antigen, but in order to conserve space in the tables of this article, an average of the titres of the immune sera will be given.

For inoculating purposes antigens prepared from the same organism were diluted in such a manner as to correspond to a suspension of 1000 million organisms per c.c., as this was found to be a safe dose for rabbits, and as it also compares very closely to the count of the broth cultures.

For agglutination purposes the bacterial suspensions were sterilized with formalin; the bacillary suspensions were made by washing the organisms from agar with physiologic salt solution containing 0·5 per cent. formalin; the organisms grown in broth were centrifugalized and taken up with same salt solution diluent containing 0·5 per cent. formalin.

The technique for the complement-fixation test and also bacteriotropin test (Neufeld) was practically the same as described by Kolmer in the 1920 edition of his text-book on 'Infection, Immunity, and Specific Therapy.'

EXPERIMENTS.

Experiment 1.—*Test of the Antigenic Value of Broth Cultures of* B. typhosus *Treated with Phenol.*

Previous experiments in this laboratory have shown that carbolic acid in strong solution (2 per cent.) will, to a certain extent, liberate antigenic substances from bacteria, although its action upon bacteria is supposed to be in the nature of a coagulation of the protein of the protoplasm of the organisms.

The present experiments were carried out as follows: To a twenty-four-hour broth culture of the organism (Rawling's strain) was added 2 per cent. phenol, and the mixture evaporated to one-tenth its original volume to rid the mixture of the phenol, after which it was diluted to original volume and centrifugalized. During the course of the experiment fractions or samples were obtained at various stages of the procedure and tested for antigenic properties.

Except where definitely stated, organisms were separated from broth cultures by means of the Sharples centrifuge. The separated organisms are designated as the sediment and the clarified fluid as the "centrifugate," which term more clearly defines this fluid than the terms "filtrate" or "supernatant fluid."

TABLE I.

Antigens.		Titre of immune sera.		
No.	Method of preparation.	Agglutination.	Complement-fixation.	Bacteriotropin.
1	Original broth cultures (1000 million organisms per c.c.)	1:18,000	1:10,000	1:2800
2	Centrifugate from antigen No. 1	1:16,000	1:10,000	1:6000
3	Sediment from antigen No. 1 diluted to original volume	1:4000	1:700	1:450
4	Original culture plus 2 per cent. phenol	1:2000	1:600	1:1200
5	Centifugate from antigen No. 4	1:1500	1:600	1:450
6	Sediment from antigen No. 4 diluted to original volume	1:750	1:1000	1:1500
7	Antigen No. 4 evaporated to 1/10 volume and diluted to original volume	1:200	1:1500	1:450
8	Centrifugate from antigen No. 7	1:200	1:7500	1:750
9	Sediment from antigen No. 7, diluted to original volume	1:300	1:2000	1:200
10	Control—agar culture suspended in salt solution. Killed with 3 per cent. kresol, 1000 million organisms per c.c.	1:14,000	1:10,000	1:3000

Discussion.—While the phenol liberated a portion of the antigenic substance from the bacterial cells, as is shown by the complement-fixation and bacteriotropin tests of antigen No. 8, it is very evident that it had, at the same time, an injurious effect upon the antigens as a whole, as the original culture and centrifugate—antigens Nos. 1 and 2, which were not treated with phenol—show antibody stimulation far superior to any other antigen. According to the bacteriotropin test the broth centrifugate gave results even superior to the original culture. This, with other evidence, as illustrated in the first two experiments and experiments to follow, to the effect that broth centrifugates contain a high percentage of antibody-stimulating substances, and the fact that the sediments from practically all of these cases gave evidence of less antibody stimulation than the centrifugates, would warrant the suggestion that the antigen is very loosely bound to the bacterial cells, and that it is not necessary to disintegrate the cells in order to obtain the antigen free in solution.

Experiment 2.—*Test of the Antigenic Value of Broth Cultures of* B. typhosus *Treated with Sodium Hydroxide.*

Sodium hydroxide has been used successfully for a number of years for the disintegration of bacteria, and it is believed by many that as a result of the destruction of the bacterial cells the endotoxic or antigenic substances are liberated. It will be recalled that Vaughan resorted to the use of absolute alcohol containing 2 per cent. sodium hydroxide for the liberation of protein from bacterial cells, and Aronson (1900) extracted the diphtheria bacillus with sodium hydroxide and precipitated the extract with acetic acid and alcohol. More recently Thomson (1921), following somewhat the method of Aronson, but using stronger solutions of alkali and acid, produced his "detoxicated"

vaccines, and Jenkins (1921), following the same line of procedure, somewhat modified, prepared his "residual" vaccines.

This experiment was carried out similarly to Experiment 1, using 1 per cent. 10 N sodium hydroxide instead of phenol, and fractions were obtained and tested as in Experiment 1.

TABLE II.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
1	Original broth culture (1000 million organisms per c.c.)	1 : 18,000	1 : 10,000
2	Centrifugate from antigen No. 1	1 : 16,000	1 : 10,000
3	Sediment from antigen No. 1 diluted to original volume	1 : 4000	1 : 700
11	Antigen No. 1 plus 1 per cent. 10 N NaOH	1 : 1500	1 : 600
12	Centrifugate from antigen No. 11	1 : 1000	1 : 400
13	Sediment from antigen No. 11 diluted to original volume	1 : 800	1 : 700
14	Antigen No. 11 evaporated to 1/10 volume and diluted to original volume	1 : 150	1 : 2000
15	Centrifugate from antigen No. 14	1 : 150	1 : 2000
16	Sediment from antigen No. 14 diluted to original solution	1 : 100	1 : 450
10	Control—agar culture suspension in salt solution, 1000 million bacteria per c.c. killed with 0·3 per cent. kresol	1 : 14,000	1 : 10,000

Discussion.—It is very evident that the antigenic properties, as represented by agglutination and complement-fixation tests, are to be found more active in the original broth culture and first centrifugate, antigens Nos. 1 and 2, than at any other stage of the procedure. It is surprising that the organisms cleared of the broth did not make a better showing, as it has generally been conceded that the antigenic properties of a broth culture of an organism of this type were to be found in a larger percentage in the bacteria than in the broth filtrate. It is also surprising that the antibody response from the broth centrifugate was greater than that of the control fraction (suspension from the agar growth).

Treatment with sodium hydroxide did not seem to aid in any way in liberating the antigenic properties of the bacteria. On the other hand, the alkali appeared to have an injurious effect upon the antigens.

The action of the sodium hydroxide upon the bacterial cells seemed to have some effect, however, as antigen No. 15, which is practically an extract, stimulated more antibody response (as indicated by the complement-fixation test) than antigens Nos. 14 or 16 which contain the cell bodies.

Experiment 3.—*An Experiment similar to Experiment 2, using Antiformin Instead of Sodium Hydroxide as a Solvent for the Bacteria.*

Antiformin is a commercial product often employed in laboratories for the purpose of disintegrating bacteria in the preparation of soluble antigens.

To a portion of the original broth culture which was used for Experiment 1 was added 1 per cent. antiformin, and the mixture evaporated to one-tenth volume and then diluted to original volume.

Fractions corresponding to those of Experiment 1 were tested by the agglutination and complement-fixation tests for antibody response.

TABLE III.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
1	Original broth cultures (1000 million organisms per c.c.). Same as for Exp. 1 :	1 : 18,000	1 : 10,000
2	Centrifugate from antigen No. 1	1 : 16,000	1 : 10,000
3	Sediment from antigen No. 1 diluted to original volume	1 : 4000	1 : 700
17	Antigen No. 1 plus 2 per cent. antiformin	1 : 1000	1 : 800
18	Centrifugate from antigen No. 17	1 : 1000	1 : 500
19	Sediment from antigen No. 17 diluted to original volume	1 : 900	1 : 600
20	Antigen No. 17 evaporated to 1/10 volume and diluted to original volume	1 : 150	1 : 600
21	Centrifugate from antigen No. 20	1 : 100	1 : 1800
22	Sediment from antigen No. 20 diluted to original volume	1 : 100	1 : 500
10	Control—agar culture suspended in salt solution; killed with 0·3 per cent. kresol; 1000 million organisms per c.c.	1 : 14,000	1 : 10,000

Discussion.—The results are very similar to those of Experiment 2. There seems to be a deterioration or weakening of the antigenic substances due to the action of the antiformin.

Again, antigen No. 21 (corresponding to antigen No. 8 of Experiment 1), as recorded by the complement-fixation test, seems to stimulate more antibody response than either antigens Nos. 20 or 22, which contain the bacterial cells or remnants of the cells.

Experiment 4.—*Repetition of Experiment* 3, *using* B. coli *instead of* B. typhosus.

Antibody formation was determined by agglutination and complement-fixation tests.

TABLE IV.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
23	Original broth culture	1 : 18,000	1 : 150
24	Centrifugate from antigen No. 23	1 : 12,000	1 : 600
25	Sediment from antigen No. 23 diluted to original volume	1 : 900	1 : 200
26	Antigen No. 23 plus 2 per cent. phenol	1 : 2000	1 : 75
27	Centrifugate from antigen No. 26	1 : 2000	1 : 200
28	Sediment from antigen No. 26 diluted to original volume	1 : 1500	1 : 75
29	Antigen No. 26 evaporated to 1/10 volume and diluted to original volume	1 : 900	1 : 100
30	Centrifugate from antigen No. 29	1 : 300	1 : 500
31	Sediment from antigen No. 29 diluted to original volume	1 : 900	1 : 150
32	Control—agar suspension in salt solution; killed with 0·3 per cent. kresol; 1000 million organisms per c.c.	1 : 3000	1 : 100

Discussion.—In the agglutination experiment the original broth culture gave the best results, followed by the broth centrifugate antigen No. 24. Again, the sedimented bacteria, antigens Nos. 25, 28 and 31, gave less evidence of antibody response than their corresponding centrifugates, antigens Nos. 24, 27 and 30. The phenolized centrifugate showed up a little better than those prepared with sodium hydroxide or antiformin.

In the complement-fixation test the broth centrifugate, antigen No. 24, gave evidence of highest antibody response followed by the phenolized centrifugate, antigen No. 30. In all cases, as was demonstrated in the first three experiments, and will be demonstrated in later experiments, the suspensions containing bacterial cells or remnants of the cells showed comparatively low antibody response, and this is contrary to what we have heretofore been led to believe. In the agglutination test, while the broth centrifugate, antigen No. 24, did not produce a serum of as high titre as the original culture, it was much superior to that produced by the agar suspension, the control antigen, and this is a phenomenon which we have repeatedly found, and which has not been recorded by previous workers as far as we have been able to discover.

Experiment 5.—Test of Antigenic Properties of Washings of B. typhosus *Grown on Agar.*

As previous experiments have demonstrated that twenty-four-hour broth centrifugates contain antigens which will stimulate the formation of immune bodies of much higher titre than those stimulated by centrifugates of cultures five and ten days old, or by antigens composed of sedimented bacteria, it would appear as though the antigens with which we are dealing are not entirely endotoxic in nature, nor are they typical autolysates or aggressins.

It would seem as though the antigens which have given us such high antibody response, according to our method of testing, must be closely associated with the extra-cellular structure of the organisms, the ectoplasm, and that the antigen, when freed from the bacterial cells, is a fairly stable substance.

In order to obtain this antigen from the exterior of the bacterial cells, the organisms were grown on solid media for twenty-four hours, washed off with physiologic salt solution, and at once agitated for a few minutes in a mechanical shaker and immediately run through the Sharples centrifuge. This rapid handling of the organisms in salt solution precluded the disintegration of the organisms to any extent, thus preventing the liberation of the endotoxin and also the formation of autolysates.

TABLE V.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
57	Agar suspension	1 : 8000	1 : 325
58	Agar washing	1 : 11,000	1 : 500
59	Washed organisms	1 : 4000	1 : 600
60	Broth growth	1 : 7000	1 : 300
61	Broth centrifugate	1 : 8000	1 : 250
62	Broth sediment	1 : 3500	1 : 600
63	Broth sediment washed	1 : 3000	1 : 500
64	Washings from antigen No. 63	1 : 1500	1 : 80

Discussion.—The first washings from the agar growths and the first broth centrifugate gave the most favourable results as far as the agglutination tests were concerned, the immune sera stimulated by these antigens even giving higher titres than the immune sera from the agar suspension. This is a paradoxical condition that has not heretofore been noted.

According to the complement-fixation test, previous experiments were not corroborated, in that the washed organisms from both antigens mentioned above gave evidence of stimulating higher antibody response than the washings. After the second washing of the broth sediment, however, their antigenic value dropped, so that it is a question whether the first washings were complete enough, or whether the results were produced by some other cause. Later experiments, recorded on other organisms, confirm the results of the previous experiments, and show that probably the washings in this experiment were not complete enough.

As a result of this experiment it would seem plausible that the antigen found in the broth centrifugate and the agar washings is probably not of the endotoxic variety, but may be associated, as we have suggested before, with the ectoplasm of the bacterial cells. We may be justified in referring to this antigen, therefore, as an " ecto-antigen."

Experiment 6.—*Test of the Antigenic Properties of Various Pneumococcus Antigens by means of Agglutination and Complement-fixation Tests.*

These antigens were prepared from broth cultures similar to those of Experiment 3, using 2 per cent. phenol to produce a dissolution of the organisms. Fractions were taken at several stages of the procedure and tested for antigenic properties.

Agar washings were prepared according to the method described for the preparation of *B. typhosus* agar washings, and compared with the other fractions.

TABLE VI.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
65	Broth culture	1 : 80	0
66	Centrifugate from antigen No. 65	1 : 70	0
67	Sediment from antigen No. 65 diluted to original volume	0	0
68	Antigen No. 65 plus 2 per cent. phenol, evaporated, diluted to original volume	0	1 : 100
69	Centrifugate from antigen No. 68	1 : 30	1 : 115
70	Sediment from antigen No. 68 diluted to original volume	0	1 : 125
71	Agar washings	1 : 60	1 : 60

Discussion.—The agglutination tests showed that the broth cultures and the first centrifugate from the broth cultures and the agar washing gave evidence of highest antigenic properties, while the three fractions treated with the phenol gave the best results with the complement-fixation test.

According to both tests the phenolized fraction, antigen No. 69, and the

agar washings proved to be about equal in antigenic properties, and gave on the average better results than any of the other antigens.

Experiment 7.—*Comparison of the Antigenic Properties of Various Antigens Prepared from Broth Cultures of the Pneumococcus by means of Passive Protection Tests.*

The antigens tested were the same as those in Experiment 6, with the exception of the agar washings. These were not prepared at the time.

In carrying out the experiment, white mice were injected with the immune sera together with the live cultures, 0·2 c.c. of serum being injected intraperitoneally with varying dilutions of a broth culture of the pneumococcus, the M.L.D. of which was 0·000,000,001 c.c. Type 1 pneumococcus was used for the test.

TABLE VII.

Antigens. No.	Method of preparation.	Amount of immune serum injection.	No. of M.L.D. of culture neutralized.
72	Broth culture	0·2 c.c.	10,000
73	Centrifugate from antigen No. 72	,,	10,000
74	Sediment from antigen No. 72	,,	10,000
75	Antigen No. 72 plus 2 per cent. phenol, evaporated to 1/10 volume. Diluted to original	,,	10,000,000
76	Centrifugate from antigen No. 75	,,	100,000,000
77	Sediment from antigen No. 75 diluted to original volume	,,	10,000,000

Discussion.—While all of the fractions or antigens produced antisera, giving a high grade of protection against a virulent culture of the pneumococcus, it is apparent that the phenolized antigens possess by far the greatest antigenic properties. This is fairly positive evidence that phenol in some way acts as an antigenic extractive of this particular organism. The protection offered by the sera produced by these last three antigens, especially antigen No. 76, the phenolized centrifugate, is equalled only by that produced by the recognized therapeutic antipneumococcic serum obtained from the horse by a process of hyperimmunization.

Experiment 8.—*Test of the Immunizing Value of Agar Washings of the Pneumococcus by means of Active Protection Tests.*

Agar washings of Type 1 pneumococcus were obtained by a method similar to that described for the preparation of the washing from *B. typhosus* and injected into white mice, carrying out two methods of immunization.

Lot No. 1 were immunized by two subcutaneous injections of 0·5 c.c. of the antigen seven days apart and were given the virulent culture intraperitoneally one week after the last injection, following the method of Perlzweig and Steffen (1923). Lot No. 2 were given four injections of 0·5 c.c. each three days apart, and, similarly to Lot No. 1, received the virulent culture one week after the last injection.

TABLE VIII.

Antigen No. 78.	Culture.	Results.
	Lot No. 1.	
2 injections	·0001 c.c.	Lived.
	·001 „	„
	·01 „	Died.
	·1 „	„
	Lot No. 2.	
4 injections	·00001 c.c.	Lived.
	·0001 „	Died.
	·001 „	„

M.L.D. of culture 0·000,000,001 c.c.

Discussion.—Mice of Lot No. 1 were protected against one million fatal doses of the culture, while those of Lot No. 2 were protected against ten thousand fatal doses.

This experiment confirms previous ones to the effect that the antigen may be an ecto-antigen, and tends to throw some doubt on the idea that the antigen of this organism is entirely endotoxic in nature. It will be recalled that the previous experiment would tend to lead one to believe that the antigen is, in a large part at least, endocellular in nature.

Experiment 9.—*Comparison of Various Streptococcus Antigens.*

In order to save time as well as animals, all antigens which have, in previous experiments, given evidence of low antigenic power have been eliminated in this and further experiments. The antigens which have been considered important from a scientific and practical standpoint and which will be further tested are the broth culture, first centrifugate, phenolized centrifugate, the agar suspension and agar washings.

TABLE IX.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
79	Broth culture	1 : 500	1 : 10
80	Centrifugate from antigen No. 79	1 : 600	1 : 10
81	Centrifugate corresponding to antigen No. 75 of Exp. 7	1 : 600	1 : 25
82	Agar suspension	1 : 800	1 : 60
83	Agar washings	1 : 800	1 : 50

Discussion.—The centrifugates and agar washings show high antigenic properties, the centrifugates giving evidence of higher antibody response than the broth culture, while the agar washings equal in antigenic value the agar suspension.

Experiment 10.—*Test of Gonococcus Antigens.*

The broth culture, broth centrifugate, phenolized centrifugate, agar suspension and agar washings were tested, in this experiment, for antigenic properties.

TABLE X.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
84	Broth growth	1 : 400	1 : 10
85	Centrifugate from antigen No. 84	1 : 400	1 : 10
86	Phenolized centrifugate	1 : 100	1 : 25
87	Agar suspension	1 : 500	1 : 100
88	Agar washings	1 : 600	1 : 100

Discussion.—Again the broth centrifugate and agar washings show up well as compared with the other antigens.

The phenolized centrifugate in this case did not make as good showing as in the previous experiment.

Experiment 11.—*Comparative Tests of the Immunizing Response of Agar Washings and Suspensions of* B. pertussis.

As the pertussis bacillus is grown with difficulty in liquid media, only those antigens prepared from growths on solid media were tested for antibody stimulation.

The antigenic value of agar washings was compared with that of a bacterial suspension in salt solution.

In preparing the antigens, the organisms were taken off the media with salt solution and divided into two parts—one for the bacterial suspension, and one immediately shaken and passed through the Sharples centrifuge.

TABLE XI.

Antigen.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
89	Bacterial suspension	1 : 5000	1 : 625
90	Agar washing	1 : 3000	1 : 1000

Discussion.—The antigenic value of the washings proved in this test to be about equal to that of the bacterial suspension.

Experiment 12.—*Test of the Comparative Value of Bacterial Antigens for Stimulating Polyvalent Antibody Response.*

The typhoid-paratyphoid combination, as found in the regular typhoid-paratyphoid vaccine, was followed in testing the comparative antigenic values of bacterial suspensions, agar washings and broth centrifugates.

As a control the combined bacterial suspension (antigen No. 122) was represented by the regular typhoid-paratyphoid vaccine prepared according to the United States Hygienic Laboratory Regulations, which demand that the vaccine be sterilized by subjecting it to a temperature of 52°C. for one hour. This, no doubt, has a tendency to lower its antigenic value.

TABLE XII.

Antigens.		Titre of immune sera.	
No.	Method of preparation.	Agglutination.	Complement-fixation.
112	Agar suspensions of *B. typhosus*	1 : 12,000	1 : 500
113	„ washings	1 : 16,000	1 : 675
114	Broth growth	1 : 12,000	1 : 250
115	„ centrifugate	1 : 12,000	1 : 675
116	Agar washings of *B. paratyphosus A*	1 : 6000	1 : 100
117	Broth centrifugate „	1 : 4000	1 : 50
118	Agar washings of *B. paratyphosus B*	1 : 16,000	1 : 850
119	Broth centrifugate „	1 : 600	1 : 100
120	Mixture of antigens Nos. 113, 116 and 118, in proportion corresponding to antigen No. 122, control antigen:		
	Serum tested against *B. typhosus*	1 : 16,000	1 : 750
	„ „ *B. para. A*	1 : 2000	1 : 75
	„ „ *B. para. B*	1 : 20,000	1 : 800
121	Mixture of antigens Nos. 115, 117 and 119, in proportion corresponding to antigen No. 120:		
	Serum tested against *B. typhosus*	1 : 10,000	1 : 75
	„ „ *B. para. A*	1 : 3000	1 : 60
	„ „ *B. para. B*	1 : 12,000	1 : 750
122	Regular typhoid-paratyphoid vaccine (control):		
	Serum tested against *B. typhosus*	1 : 6000	1 : 100
	„ „ *B. para. A*	1 : 5000	1 : 50
	„ „ *B. para. B*	1 : 8000	1 : 250

Discussion.—In all instances the agar washings gave evidence of greatest antibody response, even greater than the regular typhoid-paratyphoid vaccine. It should be noted also that the broth centrifugate combination stimulated a higher antibody response than the regular vaccine.

This at once suggests the question of the relative value of agar washings and bacterial suspensions for prophylactic immunization and, also, as to the value of the agglutination test for determining antibody response, inasmuch as the standardization of typhoid-paratyphoid vaccine, according to the United States Government, is based on the agglutination test. If the agglutination test is a criterion of antigenic value, then agar washings are superior to the regular vaccine for prophylactic purposes. If the agglutination test is not a criterion it should not be used as a means of measuring the value of a vaccine

Experiment 13.—*Comparison of Experimental Antigens with Various Typhoid-Paratyphoid Antigens Used for Therapeutic Purposes.*

The products, with the exception of the experimental antigens, were obtained on the open market, and were diluted to the same corresponding strength with salt solution, according to the strength of the antigen as given on the label, based on the number of organisms per cubic centimetre.

This experiment gives the comparative values of the polyvalent antibody stimulation of the combined antigen, as each antigen was tested for evidence

of immune body response of the individual organism composing the combined antigens.

TABLE XIII.

Antigens.			Titre of immune sera.	
No.	Method of preparation.		Agglutination.	Complement-fixation.
125	Typhoid vaccine		1:9000	1:1000
122	Typhoid-paratyphoid vaccine comb.	*B. typhosus*	1:6000	1:100
		B. para. A.	1:5000	1:50
		B. para. B.	1:8000	1:250
120	Typhoid-paratyphoid washings	*B. typhosus*	1:16,000	1:750
		B. para. A.	1:2000	1:750
		B. para. B.	1:20,000	1:750
121	Typhoid-paratyphoid broth centrifugate	*B. typhosus*	1:10,000	1:175
		B. para. A.	1:3000	1:60
		B. para. B.	1:12,000	1:750
126	Typhoid glycerol vaccine	*B. typhosus*	1:6000	1:90
127	Typhoid serobacterin	*B. typhosus*	1:5000	1:75
128	Detoxicated T.A.B. vaccine	*B. typhosus*	1:2000	1:25
		B. para. A.	1:20	1:5
		B. para. B.	1:200	1:35
129	Residual T.A.B. vaccine	*B. typhosus*	1:2000	1:25
		B. para. A.	1:20	1:5
		B. para. B.	1:200	1:35
130	Antigen prepared as phenolized centrifugate	*B. typhosus*	1:20	1:20
		B. para. A.	1:100	1:20
		B. para. B.	1:50	1:75

Discussion.—This test can only approximate at best the real comparison of the antigenic values of the various antigens, as the dilutions under the conditions could not be accurately determined. An advantage was also given to the experimental products, as they were all prepared from strains of organisms homologous to the antigens of the agglutination and complement-fixation tests. In all probability the organisms from which the various stock preparations were prepared were from strains different from those of the test antigens.

According to the results of the agglutination tests, however, the antigen composed of the agar washings produced the highest antibody response followed by the broth centrifugate. The regular bacterial vaccine, glycerol vaccine and serobacterin are third in order, with a much lower but about an equal antigenic value between the detoxicated and residual vaccines. The phenolized antigen gave evidence of relatively low antibody response.

Experiment 14.—*Comparison of Various Gonococcus Antigens used for Therapeutic Purposes with Certain Experimental Antigens.*

This experiment was carried out in a manner similar to that of Experiment 13, and, therefore, entails the same limitations as to accurate deductions.

Only the immune body response to the gonococcus antigen was tested in the combined antigens, as it was not considered important at this time to test for response to the antigens of the other organisms.

TABLE XIV.

No.	Antigens. Method of preparation.	Titre of immune sera. Agglutination.	Complement-fixation.
131	Agar washings of gonococcus	1 : 2000	1 : 100
132	Broth centrifugate of gonococcus	1 : 400	1 : 50
133	Gonorrhœa (comb.) vaccine	1 : 900	1 : 90
134	Residual gonococcus vaccine	1 : 80	1 : 10
135	Gonococcus glycerol vaccine	1 : 100	1 : 70
136	„ serobacterin	1 : 30	1 : 50
137	Antigen prepared as phenolized centrifugate	1 : 300	1 : 50

Discussion.—Again, as with the previous experiments, the agar washings and broth centrifugate gave evidence of greater antibody stimulation followed by the combined bacterial vaccine.

The antigen prepared as a phenolized centrifugate appeared to be superior in antigenic activity to the three remaining products.

CONCLUSIONS.

Comparative tests of the antigenic value of various bacterial preparations were made, with the following results:

(1) Sodium hydroxide did not seem to aid, to any great extent, in liberating antigenic properties from typhoid bacilli. On the other hand, the alkali in the strength used appeared to have an injurious effect upon all antigens.

(2) Antiformin and phenol had about the same effect as sodium hydroxide.

(3) In all experiments the antigens were found more active in the original broth culture and first centrifugate than in any of the treated antigens.

(4) The sedimented organisms did not show up as well as the centrifugates from these organisms.

(5) Salt solution washings from twenty-four-hour agar growths of *B. typhosus* proved to be higher in antigenic properties than the corresponding bacterial suspension or the washed organisms, also higher in antigenic properties than a broth culture of the same bacterial count, the broth centrifugate as well as the sediment from the broth culture.

(6) Antigens obtained from broth filtrates and agar washings of young growths of organisms appear to originate, not from the endoplasm, as endotoxin, nor from the secretions of the organisms, as exotoxins, but from the ectoplasm of the bacterial cells. We have named these "ecto-antigens."

(7) Agglutination tests showed that the pneumococcus broth culture, broth centrifugate and agar washings were about equal in antigenic value.

(8) The pneumococcus passive protection tests exhibited some interesting results. The phenolized centrifugate stimulated the highest antibody response, followed by the phenolized broth culture and the sediment.

(9) Agar washings of the pneumococcus produced an active immunity in white mice which protected them against one million fatal doses of the organism.

(10) The streptococcus agar washings equalled the antigenic value of the bacterial suspension and was superior to the broth centrifugate, which, in turn, gave better results than the broth culture.

(11) The gonococcus agar washings gave best results, followed by the bacterial suspension. The broth centrifugate and broth culture gave readings of equal value.

(12) The bacterial suspension of the pertussis bacillus, according to the agglutination test, gave evidence of higher antibody response than the agar washings, while, according to the complement-fixation test, the reverse was true.

(13) In the production of polyvalent antibodies to the typhoid-paratyphoid group, agar washings gave evidence of greater response than that produced by a corresponding bacterial vaccine.

(14) Typhoid-paratyphoid agar washings gave evidence of higher antibody response than other antigens of similar nature used for therapeutic purposes.

(15) Gonococcus agar washings stimulated a greater antibody response than similar therapeutic antigens tested.

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